Volume 89 Number 2

Science Library

NOV 6 1945

THE BIOLOGICAL BULLETIN

PUBLISHED BY

THE MARINE BIOLOGICAL LABORATORY

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OCTOBER, 1945

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

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Vol. 89, No. 2 October, 1945

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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

STUDIES ON THE BIOCHEMISTRY OF TETRAHYMENA. IV. AMINO ACIDS AND THEIR RELATION TO THE BIOSYNTHESIS OF THIAMINE

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It was reported earlier (Kidder and Dewey, 1942) that two species of Tetrahymena were able to carry out the synthesis of thiamine, if provided with a substance found mainly in the leaves of plants. This substance was called Factor S and was found in highest concentration in alfalfa leaf meal but could not be demonstrated from materials of animal origin. Factor S was characterized by its solubility in water and alcohol (up to 75 per cent), insolubility in ether and acetone, stability to prolonged heat in the presence of either alkali or acid, and its stability to ultraviolet radiation. It was shown to be dialyzable through cellophane and not to be precipitated by the salts of heavy metals. It was shown that Tetrahymena gave optimal growth in a medium consisting of "vitamin-free" casein, salts and a heat- and alkali-treated water extract of alfalfa meal. Very little growth occurred in the absence of the alfalfa extract and the addition of thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, pimelic acid, *i*-inosital, uracil, or *p*-aminobenzoic acid either singly or in combination had no significant effect. Inasmuch as the heat- and alkali-treated alfalfa extract was certainly free of thiamine it was concluded that Tetrahymena could synthesize the thiamine required for its metabolic needs when supplied with Factor S. It was suggested that Factor S possibly acted as a catalyst necessary for the synthesis of the thiamine molecule.

It was recognized that the alfalfa extract used contained Factors I and II (Dewey, 1941; 1944) and we now know that the casein base contained Factor III (Kidder and Dewey, 1945a).

This work was criticized by Hall and Cosgrove (1944) on the basis that the "vitamin-free" casein used for the base medium was not free of thiamine. They reported growth of their strain of Tetrahymena in heat- and alkali-treated casein in the presence of thiamine and not in its absence. This criticism was shown to be invalid (Kidder and Dewey, 1944) when an extension of the earlier studies was carried out, using heat- and alkali-treated base media (casein, casein hydrolysate, gelatin, gelatin hydrolysate). It was then found that heat and alkali treatment of

[1] Aided by grants from the Morgan Edwards Fellowship Fund, the Manufacturers Research Fund for Bacteriology and Protozoology of Brown University.

whole casein produced toxic substances which could not be overcome by thiamine addition for *T. geleii* W but could to a slight extent for *T. geleii* H. In tryptophane-supplemented gelatin (Harris), however, indefinitely transplantable growth was possible after all of the thiamine had been destroyed. The addition of thiamine did not affect the generation time but did increase significantly the maximum yield and survival. The addition of heat- and alkali-treated alfalfa extract decreased the generation time and raised the maximum yield to optimal for the species, and the addition of thiamine had no significant effect. This was interpreted as meaning that gelatin possessed low concentrations of Factors I, II, and S and that the final cessation of growth was due to the depletion principally of Factor S, as the addition of thiamine did raise the maximum yield.

One of the difficulties encountered in the earlier work was the separation of Factor S from Factors I and II. The heat and alkali treatment of peptones seemed to destroy the Factor I activity, but toxic substances were produced which rendered the medium inferior for our tests. Nevertheless, it was possible to show that lead acetate precipitate (containing no factor S) from plant material could replace the heat- and alkali-destroyed fraction only if thiamine was added. This was taken to mean that peptone contained no Factor S but did contain Factor II which was stable to the treatment used for dethiaminization, and Factor I which was unstable. It was recognized that little more could be done until active preparations of Factors I II could be obtained which were essentially free of both Factor S and toxic materials.

Recently we have been able to obtain such a preparation and it has been possible to test the activity of Factor S. This work, to be reported here, while confirming our earlier conclusions on thiamine synthesis, has forced us to alter our original theory concerning the role of Factor S in the metabolic activities of Tetrahymena.

Material and Methods

The organism used in the present study was the ciliated protozoan *Tetrahymena geleii* W, which is the strain used in the previous studies on thiamine synthesis (Kidder and Dewey, 1942; 1944). All work was done with pure (bacteria-free) cultures. The ciliates were grown in 2 ml. quantities of media in Pyrex tubes according to the technique described elsewhere (Kidder and Dewey, 1945b). All media, made with water twice distilled over permanganate in an all-Pyrex still, were adjusted to give a final pH of 6.8–7.0 and sterilization was by autoclaving. Serial transplants were made and results are recorded only after the third transplant. Transplants were made at 72 hour intervals using a bacteriological loop delivering approximately 0.008 ml. of fluid. Incubation was at 25° C. Population densities were determined by the direct counting technique (Kidder, 1941). All glassware used in this investigation was made chemically clean with sulfuric-dichromate solution, thoroughly rinsed and air dried before use.

In order to eliminate the possibility of cotton fibers contributing substances to the medium, Pyrex wool plugs were used extensively. It was found helpful to flame the protruding ends of the plugs until a thin crust had formed to eliminate the annoying strands inevitably present in this type of plug. This treatment fuses enough of the Pyrex strands to cause the plugs to hold their shape and increases appreciably the ease with which they may be handled.

Two types of base media were used for most of this work. One was 0.5 per cent hydrolyzed Eastman purified calfskin gelatin (Lot no. *144*). This hydrolysate was prepared by refluxing 100 gr. of gelatin in one liter of 25 per cent H_2SO_4 for 5 hours, removing the sulfate as $BaSO_4$ and reducing to the required concentration. Hydrolysate prepared with HCl was also used and the two were similar in every way. The gelatin hydrolysate was supplemented in all cases with 0.01 per cent *l*(−)-tryptophane and (with one exception to be noted later) with 0.02 per cent *dl*-valine. This base medium will be referred to as EGH.

The second type of base medium employed was a mixture of the eleven amino acids found to give optimum growth for this strain of *Tetrahymena geleii* (Kidder and Dewey, 1945a). These amino acids with the concentration in mg. per cent of each were as follows: *l*-(+)-arginine monohydrochloride—82; *l*(−)-histidine monohydrochloride—10; *dl*-isoleucine—35; *dl*-leucine—35; *dl*-lysine—60; *dl*-methionine—34; *dl*-phenylalanine—14; *dl*-serine—4; *dl*-threonine—20; *l*(−)-tryptophane—10; *dl*-valine—20. This base medium will be referred to as 11 AA. The sources of the amino acids used have been given elsewhere (Kidder and Dewey, 1945b).

Inasmuch as our primary concern was with thiamine all media were made up to contain other known growth factors, minerals and sugar to insure against limiting factors outside the scope of this investigation. Accordingly to our base media the following were always added:

	mg./ml.
Difco bacto dextrose	2.00
$MgSO_4 \cdot 7H_2O$	0.10
K_2HPO_4	0.10
$CaCl_2 \cdot 2H_2O$	0.05
$FeCl_3 \cdot 6H_2O$	0.00125
$MnCl_2 \cdot 4H_2O$	0.00005
$ZnCl_2$	0.00005
	Micrograms/ml.
Biotin methyl ester	0.00005
Calcium pantothenate	0.10
Nicotinamide	0.10
i-Inositol	1.00
Choline chloride	1.00
p-Aminobenzoic acid	0.10
Pyridoxine hydrochoride	0.10
Uracil	0.10
Folic acid[2]	0.01
Riboflavin	0.10

The sources of the salts and growth factors have been given earlier (Kidder and Dewey, 1945b).

Our preparation containing Factors I, II, and III was made from Liver Fraction L.[3] Fifteen grams of Liver Fraction L was dissolved in 750 ml. of distilled water, adjusted to pH 4.5, and extracted continuously for 96 hours in a liquid-liquid extracting apparatus (Wilson, Grauer, and Saier, 1940) with 750 ml. of *n*-butyl alcohol. The extracted material was freed of butyl alcohol, neutralized and

[2] Folic acid concentrate with a "potency" of 5000, furnished through the courtesy of Dr. R. J. Williams.

[3] Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

the volume reduced to 300 ml. This was designated 12L, and was found to contain adequate amounts of Factors I, II, and III. The pH of this preparation was adjusted to 9.5–10.5 with NaOH and heated in the autoclave at 123° C. for one hour for dethiaminization. This preparation will be designated 12L1, which was found to be free of Factor S activity. 12L1 was used as a supplement in a final concentration of 1 : 20.

Preparations containing Factor S were obtained from alfalfa meal. Water extract of alfalfa, as previously described (Kidder and Dewey, 1942; 1944), was heated in the autoclave at 123° C. for one hour at pH 9.5–10.5 to insure the destruction of thiamine. This preparation, designated A, was used in a final concentration of 1 : 10.

Results

When Liver Fraction L is heated with alkali to destroy thiamine, changes take place which make it inferior as a source of supplementary factors for Tetrahymena. The addition of thiamine does not completely overcome these toxic effects, although the inhibition is less than that produced when proteose-peptone is dethiaminized. It was found, however, that toxic materials were not produced upon heating provided the Liver Fraction L was extracted previously with butanol. The butanol extraction was used originally for the removal of pyridoxin and riboflavin (to be reported in detail later).

Table I

Growth in EGH and 11 AA with and without added Factor S from dethiaminized alfalfa extract (A) and with and without added thiamine. All tubes contain 12L1 and the numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

Base	Additions			
	0	Thiamine	A	A + Thiamine
EGH	3,100	305,000	75,000	290,000
11AA	120,000	310,000	165,000	300,000

It was found that optimum growth resulted when a gelatin hydrolysate medium (with tryptophane, valine, and ten known growth factors), referred to as EGH, was supplied with 12L1 and thiamine, and very low growth occurred when the thiamine was omitted. The 12L1 was low in Factor S yet contained adequate amounts of Factors I, II, and III. This offered the opportunity to test the mode of action of Factor S, which could now be supplied from plant material without reference to the amounts of essential growth factors. Accordingly tests were set up using both EGH and 11 AA as base media, both supplemented with 12L1. To these base media were added various combinations of dethiaminized alfalfa extract (A) and thiamine. The results which were expected, namely the failure of growth unless either thiamine or Factor S was present, were not realized in 11 AA. Table I shows that very little growth occurred in the media based on EGH unless thiamine or Factor S was supplied but relatively good growth was obtained in the amino acid mixture in the absence of both. It will also be noted that thiamine is much more stimulatory, under these conditions, than is Factor S.

It was apparent from the foregoing results that the ability of Tetrahymena to synthesize thiamine was not dependent on the presence of Factor S when 11 AA was used as the base medium. This led to the conclusion that either some amino acid or combination of amino acids in the gelatin hydrolysate was blocking the synthetic mechanisms or that materials in the 12L1 were causing the block, the latter block being removed by some combination of the pure amino acids not present in the gelatin hydrolysate. The first of these possibilities was tested by making up an amino acid mixture based exactly on the published analysis for gelatin, but adding both tryptophane and valine (indispensable for this species). The ciliates behaved in this synthetic gelatin hydrolysate just as they had in 11 AA, so it was apparent that the first of the possibilities was untenable. The only known difference between the synthetic gelatin hydrolysate and EGH from a qualitative point of view was the inclusion in the former mixture of synthetic unnatural isomers (in the *dl* form, because of availability) of a number of the amino acids.

The addition of 11 AA to EGH plus 12L1 resulted in good growth without the addition of either thiamine or Factor S. This led us to test the effect of omitting each of the 11 amino acids singly from the 11 AA added to EGH. These results were inconclusive as fair growth occurred in all tubes. This was taken to mean that more than one of the 11 amino acids could counteract the inhibition to thiamine synthesis.

TABLE II

Growth of EGH with the addition of varying concentrations of racemic mixtures of amino acids. All tubes contain 12L1. The numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

Amino acid	Concentration of amino acid added (mg./ml.)					
	0.1	0.3	0.5	0.8	1.0	Control, nothing added
dl-phenylalanine	84,000	80,000	82,000	94,000	101,000	3,800
dl-methionine	92,000	8,600	8,000	6,000	7,800	
dl-serine	58,000	82,000	97,000	114,000	110,000	
dl-norleucine	31,000	11,500	0	0	0	
dl-aspartic acid	21,000	46,000	51,000	87,000	62,000	
dl-isoleucine	11,500	56,000	70,000	97,500	60,000	
dl-lysine monohydrochloride	4,500	15,000	58,000	61,000	78,000	
dl-threonine	6,000	31,000	42,000	66,000	81,000	
dl-homcystine	10,500	33,000	11,000	8,000	6,400	
dl-alanine	3,000	4,200	26,000	11,000	4,500	
dl-glutamic acid	6,500	7,500	12,500	21,000	37,000	

The next set of experiments was designed to determine whether or not the addition of single amino acids to the gelatin hydrolysate medium could counteract the inhibition to thiamine synthesis. Arbitrary amounts of each of nineteen amino acids were added to EGH. Thiamine synthesis occurred to a marked degree in some of the tubes, moderately in others and very little in some. In all cases where the inhibition was not removed the amino acid used was in its natural form while those amino acids which were most effective were synthetic.

This set of experiments was repeated using varying concentrations of the synthetic amino acids and some of the results are given in Table II. It will be seen that the effectiveness of the amino acids in releasing the inhibition of thiamine synthesis varied with the amino acid and the concentration. Phenylalanine was the most effective throughout the range of concentrations used while methionine was most effective in the lowest concentration. Norleucine was moderately effective at a concentration of 0.1 mg. per ml. but was toxic at 0.5 mg. per ml. or higher. These results indicated that the unnatural isomers were in some way able to release the inhibition of thiamine synthesis. It seemed more probable that the ratio between the two isomers was not the explanation, as some release of inhibition was found with some of the nonsynthetic amino acids. It is known that in the preparation of amino acids from natural sources some racemization is likely to occur and this might account for the small amount of activity.

TABLE III

Comparison of the effect of the natural isomer (1+) and the unnatural isomer (1−) of isoleucine, added to EGH + 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

Amino acid	Concentration of amino acid added (mg./ml.)					
	0.1	0.3	0.5	0.8	1.0	Control, nothing added
l(+)-isoleucine	7,800	4,100	6,300	9,800	11,500	3,100
l(−)-isoleucine	42,000	91,000	81,000	68,000	21,000	

This was shown to be the probable explanation by two sets of experiments. We had samples of natural *l*(+)-isoleucine, unnatural *l*(−)-isoleucine and synthetic *dl*-isoleucine. A comparison of the figures for *dl*-isoleucine in Table II with those in Table III shows that *l*(−)-isoleucine is effective in approximately one half the required concentration of *dl*-isoleucine. This is what is to be expected if only the

TABLE IV

Comparison of the effect of the natural isomer (1−) and the racemic mixture (dl) leucine, added to EGH + 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

	Concentration of amino acid added (mg./ml.)					
	0.1	0.3	0.5	0.8	1.0	Control, nothing added
l(−)-leucine	2,500	3,500	10,500	14,000	13,500	3,800
dl-leucine	4,100	5,500	26,000	29,000	31,000	

unnatural isomer is effective in the removal of thiamine synthesis inhibition. The effectiveness of *l*(+)-isoleucine is low and increases with the concentration. This could be due to the occurrence of some racemization during its preparation.

When natural leucine was compared to *dl*-leucine the former was found to be less effective in the release of the synthesis inhibition (Table IV). The difference

here, however, was not as marked, as the natural form appears to contain a considerable quantity of racemic mixture and the synthetic leucine is rather low in activity. It should be noted that we used Kahlbaum *dl*-leucine as this was found previously (Kidder and Dewey, 1945b) to be free of isoleucine, a common contaminant of many brands of synthetic leucine (Hegsted and Wardwell, 1944).

Inasmuch as EGH contained added *dl*-valine it was thought advisable to determine whether the unnatural isomer of this amino acid might be responsible for the ability of the ciliates to grow at all without added thiamine, Factor S or unnatural isomers of amino acids (see controls in Tables I–IV). Accordingly EGH minus valine was tested with varying concentrations of *dl*-valine with and without thiamine. Table V shows that without thiamine, very little growth occurs with no

TABLE V

Effect of the addition of dl-valine to EGH (minus valine). All tubes contain 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

	Concentration of dl-valine (mg./ml.)						
	0	0.05	0.1	0.3	0.5	0.8	1.0
Minus thiamine	150	2,500	4,000	7,500	16,000	37,000	45,000
Plus thiamine	190,000	210,000	265,000	310,000	305,000	325,000	315,000

added valine, and that the inhibition to thiamine synthesis is counteracted more effectively the higher the concentration of added *dl*-valine. With added thiamine, however, the addition of valine had little effect. This indicates that the sample of gelatin used differs from our previous sample of Eastman de-ashed gelatin in that it contains nearly optimum amounts of natural valine for this species. It had previously been found (Kidder and Dewey, 1945b) that Eastman de-ashed gelatin would not support growth of *Tetrahymena geleii* W without added valine, even in the presence of thiamine. The fact that transplantable, though very low, growth occurs without the addition of any unnatural isomers of amino acids may mean that the inhibition to thiamine synthesis is never complete or that some racemization of the amino acids has occurred during hydrolysis.

When thiamine was added (0.1 micrograms per ml.) to any of the above described combinations, growth was always raised to approximately 300,000 ciliates per ml. Thiamine, therefore, although it can be synthesized by the ciliates, is very active as a stimulatory substance. It was of interest and importance to determine the amount of stimulation produced by different concentrations of thiamine when added to EGH plus 12L1; EGH plus 12L1 and one of the active amino acids; EGH plus 12L1 and Factor S; and 11 AA plus 12L1. Figures 1–4 show a summary of the activity of various concentrations of thiamine. The lowest concentration tested was 0.005 millimicrograms per ml. and in every case this amount gave significant stimulation. The stimulation was roughly proportionate to the concentration up to 0.001 micrograms per ml. In all cases, after this point, the amount of growth was increased more gradually but reached approximately the 300,000 level at 0.01 micrograms per ml. of thiamine when inhibition to thiamine synthesis was absent or removed. Ten times this amount of thiamine was required to raise

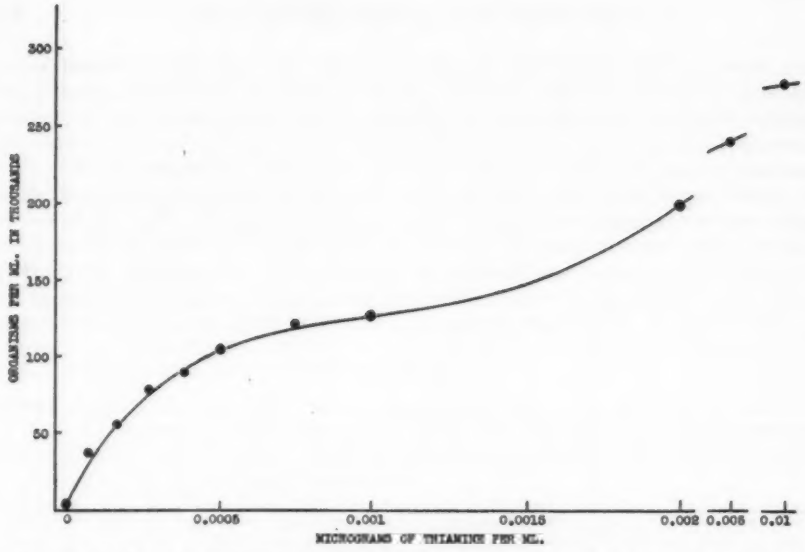

FIGURE 1. Curve of population densities at various concentrations of thiamine hydrocloride with gelatin hydrolysate (EGH) and dethiaminized butanol extracted Liver Fraction L (12L1) as base. The concentration of organisms was determined from the third transplant after 72 hrs. of growth.

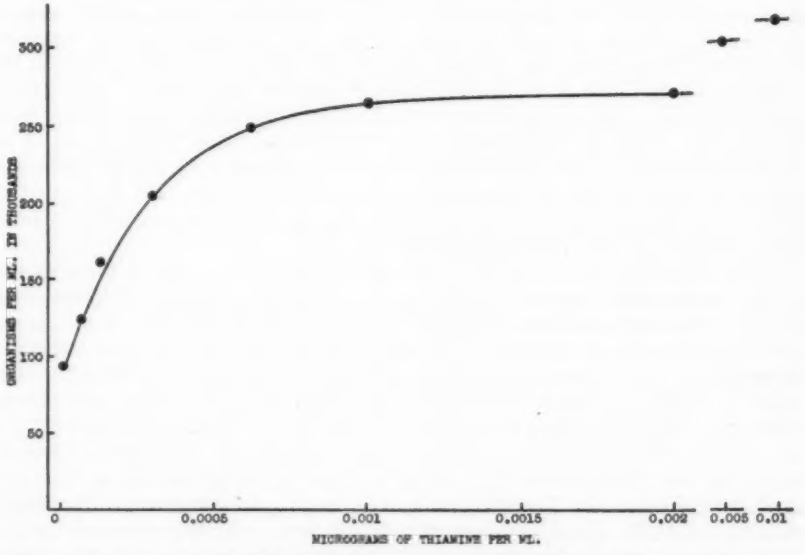

FIGURE 2. Curve of population densities at various concentrations of thiamine hydrochloride with EGH, 12L1 and *dl*-serine (0.5 mg./ml.) as base. Third transplant determinations after 72 hrs. of growth.

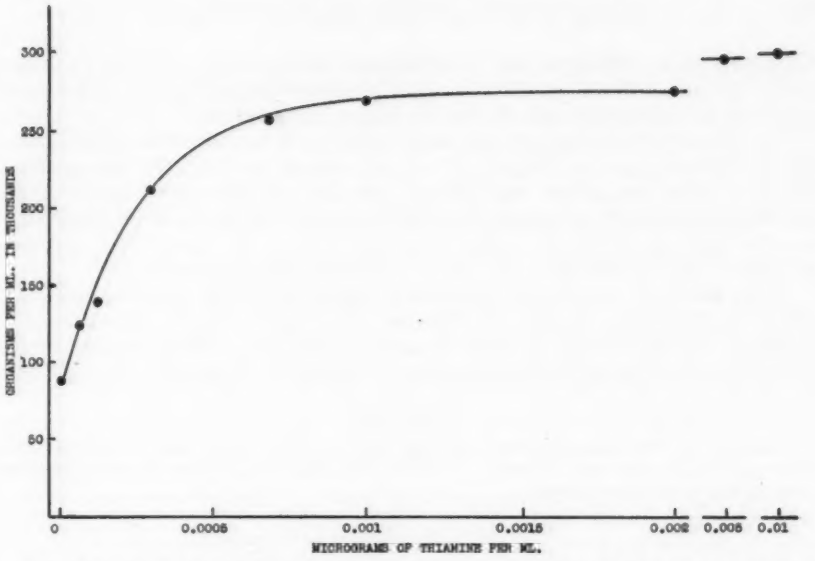

FIGURE 3. Curve of population densities at various concentrations of thiamine hydrochloride with EGH, 12L1 and dethiaminized alfalfa extract (A) as base. Third transplant determinations after 72 hrs. of growth.

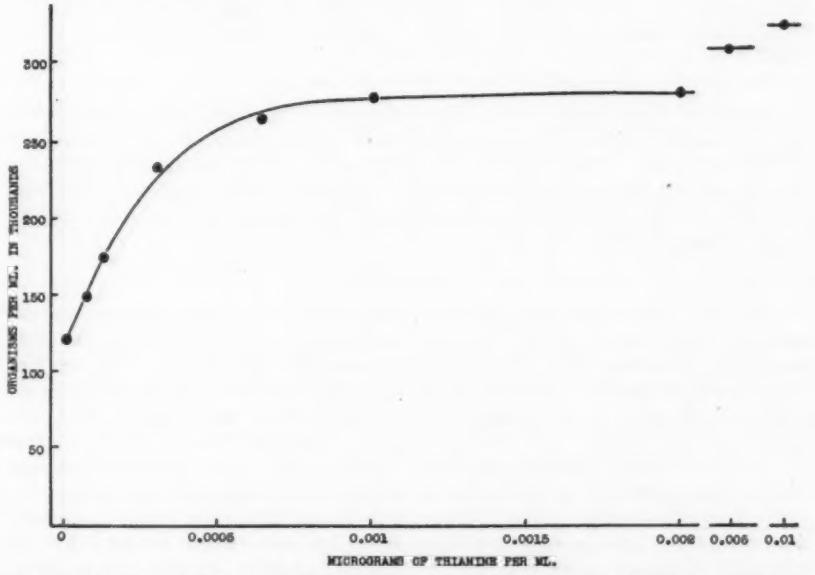

FIGURE 4. Curve of population densities at various concentrations of thiamine hydrochloride with the amino acid mixture (11 AA) and 12L1 as base. Third transplant determinations after 72 hrs. of growth.

the population to 300,000 per ml. where inhibition was pronounced (Fig. 1). These results show that Tetrahymena is far more sensitive to thiamine below a concentration of 0.001 micrograms per ml. than to higher concentrations.

An interesting and perhaps important point to be noted in the data shown in Figure 1 is the inflection which occurs in the curve above the 0.001 microgram per ml. level. The reasons for this inflection are not clear, although it seems possible that thiamine may be performing a double role where inhibition is pronounced. It may be supplying the vitamin needs of the organisms at the lower levels and acting to remove other inhibitions to growth as the concentrations increase.

Only the intact molecule of thiamine is capable of giving optimum stimulation. When the pyrimidine portion of thiamine (2-methyl-5-ethoxymethyl-6-amino pyrimidine) [4] or the thiazole portion (4-methyl-5-beta-hydroxyethyl thiazole) [4] were added separately or together some release of inhibition occurred. Table VI shows

TABLE VI

Growth in EGH plus 12L1 with varying concentrations of the thiazole and pyrimidine components of the thiamine molecule. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

	Concentration (micrograms/ml.)					
	0.001	0.005	0.01	0.1	0.5	Control, nothing added
Thiazole	3,000	8,400	20,000	1,400	1,000	2,900
Pyrimidine	1,200	5,300	17,500	3,200	2,600	
Thiazole and pyrimidine (total conc.)	2,500	11,000	24,000	2,400	2,500	

the results of these experiments. Both thiazole and pyrimidine produce stimulation in low concentrations but are mildly toxic at concentrations of 0.1 micrograms per ml. or higher. Thiazole and pyrimidine behave much the same as Factor S or the unnatural isomers of the amino acids, although to a less degree. They appear to cause the release of the thiamine synthesis inhibition and are themselves inhibitory in high concentrations.

DISCUSSION

It appears from the foregoing results that there are substances present in natural materials which can block the synthetic mechanisms of Tetrahymena. Under the conditions of our experiments this blocking occurred specifically in the mechanism or mechanisms for the synthesis of the thiamine molecule. That this ciliate can synthesize thiamine, as was pointed out earlier (Kidder and Dewey, 1942; 1944) cannot be doubted, when the blocking substance is absent or the block is released. In our earlier work (Kidder and Dewey, 1942; 1944) where dethiaminized alfalfa extract was used as the supply of Factors I and II (Factor III was present in the casein and gelatin preparations; Kidder and Dewey, 1945a), it might be questioned whether the growth obtained in the absence of thiamine might be the result of no inhibitory substance rather than the presence of Factor S. However, it must be re-

[4] Both the thiazole and the pyrimidine used were furnished through the courtesy of Dr. George W. Lewis and Merck and Co.

membered that the addition of alfalfa extract to EGH plus 12L1 (which contains the inhibitory substance) removed the block. Whatever Factor S is, it is able to release the block to thiamine synthesis. But it is also seen that the unnatural isomers of the amino acids can act in a similar manner, so this reaction is far from specific as to counteracting substances. It was formerly proposed (Kidder and Dewey, 1942) that Factor S might act as a catalyst to the reaction wherein the thiamine molecule was synthesized. This hypothesis appears to be no longer tenable.

It does not seem likely that Factor S is, in reality, nothing more than racemic amino acids, for two reasons. If enough racemization occurred during the heat treatment of the alfalfa extract to account for the activity found then the same amount of racemization should have taken place in the heat treatment of 12L to produce 12L1. It was found, moreover, upon assaying the alfalfa extract for the indispensable amino acids for Tetrahymena that it did not contain enough of any one of the ten to support growth, when used in the concentration employed here. But a similar assay of 12L1 demonstrated almost optimum amounts of lysine; approximately half optimal amounts of arginine, threonine, and valine; and traces of histidine, isoleucine, leucine, and phenylalanine. It seems at present that Factor S represents some material present in alfalfa and the leaves of other plants (Kidder and Dewey, 1942), the activity of which is shared by the unnatural isomers of many of the amino acids.

The relation of amino acids to the ability of organisms to synthesize vitamins has been pointed out before. Snell and Guirard (1943) showed that alanine could replace pyridoxine for *Streptococcus fecalis* R (*S. lactis* R) and that alanine functioned to counteract the toxicity of glycine. It does seem strange, however, that the unnatural isomers appear to function in the release of thiamine synthesis inhibition for Tetrahymena. In nature this organism, being largely a bacteria feeder, probably would never be called upon to use its thiamine synthesis mechanism. The use of its ability to synthesize thiamine, therefore, is admittedly the result of artificial environmental conditions, as is also the very contact with the unnatural isomers of the amino acids.

It is apparent that, although Tetrahymena does possess the ability to synthesize thiamine, this vitamin is a potent stimulant to reproduction, size (Kidder and Dewey, 1944), and longevity (Johnson and Baker, 1943). Thiamine must, therefore, be included in complete media for this ciliate, but the amount needed appears to be less than has been previously used (Hall and Cosgrove, 1944; Kidder and Dewey, 1942; 1944).

It has been stated previously (Lwoff and Lwoff, 1938; Kidder and Dewey, 1942; 1944; Hall and Cosgrove, 1944) that heating peptones or proteins with alkali renders the media inferior for the growth of Tetrahymena. This condition could be partially counteracted for some strains by the addition of thiamine. The explanation appears now to rest in the partial destruction of serine, for we have found that if 11 AA is heat- and alkali-treated growth (with added 12L1) is very low but returns to normal with the addition of serine. Increased growth results with the addition of thiamine alone, however, indicating that this vitamin can replace serine. Or that serine (a dispensable but highly stimulatory amino acid in the presence of thiamine; Kidder and Dewey, 1945b), is one of the necessary factors for the synthesis of vitamin B_1.

The relationship which exists between the concentration of thiamine and the concentration of ciliates (Figures 1–4) might suggest that this organism would be useful for assay purposes. It would be difficult, however, to assay natural products for thiamine in a base medium composed of EGH plus 12L1, the only combination which gives a low blank, because of the likelihood of the introduction of Factor S or other materials of like nature with the substance to be assayed. Although we have not attempted to do this, it might be possible to arrange conditions so that 11 AA (Fig. 4) could be used and the values calculated as differences. Experiments directed to this end might prove valuable as the present microbiological methods are not entirely satisfactory. The majority of organisms used are stimulated by the thiamine components as well as by the whole molecule (Sarett and Cheldelin, 1944), require complex base media (Williams, 1942), or require many days of growth before results can be obtained (Robbins and Kavanagh, 1937).

Summary

1. In Eastman gelatin hydrolysate (EGH) and Factors I, II, and III from Liver Fraction L (heat- and alkali-treated to destroy thiamine) the ciliate *Tetrahymena geleii* W grows very poorly without added thiamine.

2. A mixture of amino acids (11 AA) with the dethiaminized liver fraction supports fair growth without added thiamine.

3. There appear to be substances in the liver fraction or the gelatin hydrolysate or both which specifically block the mechanism for the biosynthesis of thiamine.

4. This block can be released by Factor S from alfalfa extract or by the unnatural isomers of a number of amino acids.

5. Some release of the inhibition to thiamine synthesis is produced by a few of the natural amino acids but this is probably due to the presence of low concentrations of unnatural isomers which result from racemization during preparation.

6. The unnatural isomer of isoleucine (the only unnatural isomer available for testing) was found to be active in approximately one half the concentration of the *dl*-isoleucine.

7. Thiamine is extremely stimulatory in low concentrations.

8. The thiazole and pyrimidine components are slightly stimulatory but this stimulation appears to be due to their ability to cause some release of the thiamine synthesis inhibition.

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CERTAIN CHEMICAL FACTORS INFLUENCING ARTIFICIAL ACTIVATION OF NEREIS EGGS [1, 2]

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Introduction

Stimulation must involve physicochemical changes within cells, and the nature of such changes has been the subject of much investigation, both experimental and speculative. The process of fertilization, and the closely related process of activation in artificial parthenogenesis, have attracted special attention; and evidence has been presented for a number of interesting interpretations of this type of activation. This report concerns a group of experiments indicating a peculiar relation of picric acid to the artificial activation of the eggs of *Nereis*. The proper interpretation of these experiments might contribute to the understanding of the stimulatory process. The experiments described developed from incidental observations in connection with heat-activation, during investigations concerned with the more general question of the mode of action of heat on protoplasm.

The peculiarities of heat-activation of the unfertilized *Nereis* egg were first described by Just (1915), who was able to interpret all his data in harmony with Lillie's "fertilizin" theories. In particular, Just attributed the gradual loss of sensitivity to heat, in eggs left standing in sea water, to the diffusion from them of some fertilizin-like substance, essential to the activating process. Heilbrunn (1925) took exception to this notion, in suggesting a "colloid chemical" interpretation of heat-parthenogenesis; he believed the decrease in sensitivity to heat might be due to the gradual loss of CO_2 from the medium, resulting in alkalinization of the intracellular fluid. Heilbrunn described three experiments in which the addition of 2–4 volumes per cent of n/10 HCl to old insensitive egg-suspensions restored their original sensitivity to heat.

To reveal a possible general relation between intracellular acidity or carbon dioxide concentration and the response of cells to increased temperatures, these three observations were extended. Heilbrunn's findings were in part confirmed; but with the accumulation of large numbers of experiments, considerable variation was encountered in the response of the heat-sensitivity of the eggs to increased CO_2 concentration through acidification of the sea water. Though such pronounced effects as described by Heilbrunn were often repeatable, as many batches of eggs seemed totally unresponsive to the same treatment. In the course of testing several organic acids in this connection, however, the anomalous properties of picric acid (2, 4, 6-trinitrophenol) came to light. Extension of these properties to processes of activation by means other than heat was then attempted.

[1] This study was carried out under the direction of Dr. L. V. Heilbrunn. I gratefully acknowledge his helpful suggestions throughout the investigations, and his valuable assistance in interpreting the results.

[2] A dissertation submitted to the faculty of the Department of Zoölogy of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Materials and Methods

Ripe females of the heteronereis form of *Nereis limbata* were captured between 8 and 10 p.m., as described by Lillie and Just (1913), and kept singly or in pairs in about 200 ml. of sea water in finger bowls overnight. These were kept cool either on a salt water bench in a stream of sea water, in a refrigerator, or in a room maintained at 15° C. The last was found by far the most satisfactory in maintaining the worms with eggs intact, with apparently no ill effects. A few worms shed their eggs during the night even at this reduced temperature; these were discarded.

All experiments were begun by the transfer of one or two *Nereis* to a Stender dish containing 25 ml. of sea water. The animals were cut transversely to release the eggs, and the carcasses were quickly removed. The eggs were then concentrated toward the center of the dish by gentle rotation. Either a small quantity of an especially dense suspension of eggs was then removed to another dish, or nearly all of the supernatant fluid was withdrawn by suction, and replaced by fresh sea water. All eggs were washed in this manner through at least another change of sea water, before use. In the earliest work, samples were always tested for normalcy by treatment with sperm from males caught in the same swarm. Only very rarely was any egg ever found which did not become normally fertilized, and all samples showed well over 99 per cent germinal vesicle breakdown. Since this dependability of *Nereis* eggs is well known, and since all danger of accidental contamination of eggs with sperm was to be avoided, no such tests were made in most of the later work. Experiments were always begun on the day following capture, so that the lapse of time between capture and the first treatment was never more than 20 hours, and was only rarely over 15 hours.

All transfers of eggs were made with ordinary medicine droppers. All treatments and exposures, unless otherwise indicated, were made in a volume of 25 ml.; the egg-suspensions were of such a density that, upon settling of the eggs to the bottom, no more than half, and usually much less, of the bottom of the container was covered with a single layer. Stender dishes of about 35 ml. capacity were used except for the exposures to high temperatures; the latter were carried out in 50 ml. beakers, in which the thermal insulation is much reduced. The beakers were immersed in a small deKhotinsky constant-temperature bath to a depth 2–3 mm. above the surface of the inside liquid. The temperature of the fluid within the beakers was brought to equilibrium (at slightly less than half a degree lower than the bath temperature) before the addition of 0.3–0.5 ml. of the egg-suspension. The activating temperature used varied between 33° and 35° C., as in Just's work (1915), but was held constant to within 0.1 of a degree for any single series of tests.

Since it soon became evident that the degree of stirring had a considerable effect on the response to heat, a standard policy in this regard was always followed: upon deposition of the eggs in the warm beakers, the pipette was filled and emptied ten times successively within 4–5 seconds. This was repeated 4 minutes after the beginning of the exposure; and the beaker was removed after 5 minutes of exposure, at which time a sample of 5–8 ml. was removed to a Syracuse watch glass. In some of the earlier work, the second stirring was performed at 15 minutes, the beakers removed at 20 minutes. This exposure, which is approximately Just's optimum, yielded a better percentage of swimmers, but the shorter exposure was found to

produce the maximal amount of germinal vesicle breakdown, and was much more convenient in extended series of tests. A few tests indicated that further stirring and longer exposures led to no increase in the percentage of activation. A further trial showed that the immediate removal to Syracuse dishes was not essential; when the beakers were allowed to cool of their own accord, the residual heat did not affect the percentage of activation.

For counts of activation, 5–8 ml. of each egg-suspension were examined in a Syracuse watch glass at a magnification of about 100 ×. In certain cases involving a doubtful response, compression of the eggs between a slide and coverslip, as suggested by Heilbrunn and Wilbur (1937), and a higher magnification were necessary. The counts were made on the basis of the breakdown of the germinal vesicle, a reaction which normally occurs soon after fertilization. Counts were begun at a minimum of 2 hours after the application of the treatment in question. The advantages of the nuclear criterion are its rapidity of onset, its definite character (ordinarily admitting of easy and certain classification in counting), and its ready susceptibility to quantitative expression; the criterion is well established in work on artificial activation of this form. However, the fact should not be overlooked that the mere breakdown of the germinal vesicle in response to stimulation is seldom followed by development even approaching the normal, and there is rarely any cleavage at all. Various types of monsters are produced, mostly of the type described as due to "differentiation without cell-division," common in annelids. All of the types of stimulation used were capable of producing at least a small percentage of swimming forms, though seldom was anything like a normal trochophore seen. All counts were of 100 or 200 eggs selected by random movement of the watch glass on the stage of the microscope.

Results

Upon standing in sea water, almost all batches of eggs showed a gradual loss of sensitivity to heat, as described by Just (1915); a few, however, showed a very definite increase in sensitivity, after washing and long standing. This might perhaps be attributable to the washing away of inhibitors in the body fluids (Just, 1915); but the most pronounced of these exceptions was in a special batch in which the eggs stood in a deep layer at the bottom of a narrow container. Thus the responsible factor may have been the high CO_2 tension, in accordance with Heilbrunn's views (1925). Of several organic acids tested, however, only picric acid produced a consistent and pronounced reversal of this loss of sensitivity to heat. After a batch of eggs had become nearly or quite heat-insensitive, a bath of 15 minutes or more in sea water to which picric acid had been added to a concentration of about M/1000 (pH 6.6) was sufficient to elicit a significant response to the subsequent heat treatment in sea water. Yet the presence of the acid in the heat-treated suspensions completely prevented the activation of the eggs; if a response was to be obtained, the eggs had to be transferred back to sea water for the heat treatment.

These aspects of the action of picric acid were then tested in connection with activating agents other than heat. The agents used were ultra-violet irradiation (Heilbrunn and Wilbur, 1937), mixtures of sea water and isotonic (0.53 M) KCl (Wilbur, 1939), and mixtures of sea water and isotonic (0.35 M) sodium citrate

(Wilbur, 1941). Mixtures of KCl or citrate with sea water are denoted after the terminology of Wilbur (1941); thus a mixture of one volume of isotonic citrate and four volumes of sea water is called a "20 per cent sodium citrate mixture."

Experiments showing inhibition by picric acid of various types of activation

Heat—Of 15 experiments on the effect of picric acid on the sensitivity of eggs to heat, only one was inconsistent with the thesis that the acid inhibits the heat-activation. In these experiments, M/1000 picric acid was used, made up in sea water. Eight experiments proved useless, as the control percentages were too low to test any possible inhibition by the acid; the heat-sensitivity of these eggs is notoriously very variable between batches from different animals. The average of the seven experiments in which over 10 per cent of the control eggs responded is included in Table I, and shows a marked inhibition of the response by picric acid.

TABLE I

Inhibition by picric acid of activation of Nereis eggs by various agents

Activating agent	No. of expts.	Per cent activation in absence of picric acid	In picric acid, M/1000	
			Per cent activation	Per cent with incipient activation*
Heat	7	57	4	0
KCl mixtures	18	96	4	51
Sodium citrate mixtures	19	99	23	28

* As described on p. 147.

KCl mixtures—In fourteen experiments in which eggs were left indefinitely in a 25 per cent KCl mixture, and four similar experiments with a 50 per cent KCl mixture, almost always there was nearly 100 per cent activation in the absence of picric acid. When the acid was added to a concentration of M/1000, such activation occurred in only one instance; this case was distinctly unusual, as 74 per cent of the eggs were activated. Table I includes the averages for these experiments. However, in only 5 of the 18 tests was the breakdown of the germinal vesicle completely prevented. In the others, ordinary methods of observation (at 100 × magnification) did not reveal any certain change in appearance from the germinal vesicle stage, but a distinct nuclear outline could not be made out in many eggs. Compression of the eggs and higher magnification were necessary in counting these batches; the criterion employed was the visibility of a definite interface between the spherical nucleus and the cytoplasm. In the absence of this interface, the germinal vesicle was said to be broken down, even though no real alteration in the appearance of the egg was evident; the average percentage of the eggs so classified is presented in the last column of Table I. In these cells, the central nuclear region remained clear, the granular cortical opacity was retained, the oil droplets remained discrete and failed to migrate as in the activated eggs. None of the eggs of this type ever developed to a motile condition, or cleaved, or differentiated in any way. The appearance was as though nuclear breakdown had just barely begun when inhibition set in.

Sodium citrate mixtures—Complete or nearly complete inhibition of activation by picric acid was found in 12 of 19 experiments with citrate mixtures of 10–25 per cent. Of the other seven, two showed effective inhibition beyond the earliest stages of nuclear breakdown, as with the KCl mixtures (last column of Table I); one showed only moderate inhibition; only 4 of the 19 failed to show any significant inhibition. The averages are included in Table I. In these experiments, as in those with the KCl mixtures, the eggs were left in the activating agents indefinitely; counts were made with the eggs still in the various mixtures.

Ultra-violet irradiation—Only in relation to activation by ultra-violet rays did picric acid fail to exhibit an inhibitory effect. The presence of the acid (M/1000) in the sea water bathing the eggs did effectively prevent their activation by irradiation, but this action cannot be attributed to the effect of the acid on the eggs. Reduction of the depth of the egg-suspension to under 0.5 mm., so that the eggs are barely covered, permitted of ready activation by the rays, even in the presence of picric acid. The apparent inhibition in deeper samples is due to the absorption of the rays by the acid; the absorption spectrum of picric acid and picrates in salt solutions near neutrality (Eisenbrand and v. Halban, 1930; v. Halban and Litmanowitsch, 1941) is such that in any appreciable depth and concentration the supernatant fluid would prevent most of the active radiation from reaching the eggs, which always settle to the bottom of the dish. This interpretation is corroborated by the fact that a shield of picric acid in a quartz dish prevents any effect of ultra-violet rays on an underlying suspension of eggs in sea water.

Fertilization by sperm—Normal fertilization is completely inhibited in the solutions of acid used for the experiments above (in the range of M/1000). Addition of alkali to pH 8.0 did not affect this inhibition of fertilization. However, the removal of normally fertilized eggs to picric acid solutions within five minutes after fertilization (whether or not such solutions were alkalinized) did not appear to interfere with the normal development of the embryos; excellent survival and differentiation were obtained in the acid. Nevertheless, such embryos exhibited one outstanding anomaly: failure of the normal coalescence of the oil droplets. The oil in embryos growing in picric acid remained scattered as numerous discrete droplets; while under normal conditions these soon merge to form only a few, almost always four. The usual localization of the oil by migration (and segregation in cleavage) was not, however, altered in the course of development in picric acid solutions.

Experiments showing synergism between various activators and the removal from picric acid to ordinary sea water

Heat—Over 50 experiments tested the effect of baths in picric acid prior to exposure to heat in sea water. These showed a pronounced enhancement of the effects of the heat after the acid bath; not one showed a greater activation in the sample from sea water than in that from the acid. This relation between heat and removal from picric acid baths is shown in Figure 1. The synergistic action is evident only following the shorter baths, up to about 6 hours; since, after longer exposures to the acid, the mere removal to sea water was in itself sufficient to activate many eggs. The broken line curve in Figure 1 is made up from the combined data of all experiments involving removal of eggs from picric acid to sea water without further treatment. The other two curves on the same figure, however,

cover data from paired samples of eggs, and compare the effects of heat on eggs previously bathed in picric acid (in sea water) and on eggs from the same source not so treated.

The synergistic action was evident over a wide range of concentration of picric acid: from 10^{-4} to just over 10^{-3} M. The effects increased with increasing concentration, but above M/1000 the results became less reliable, so that M/1000 was used regularly, and is the only concentration for which data are reported. It is evident

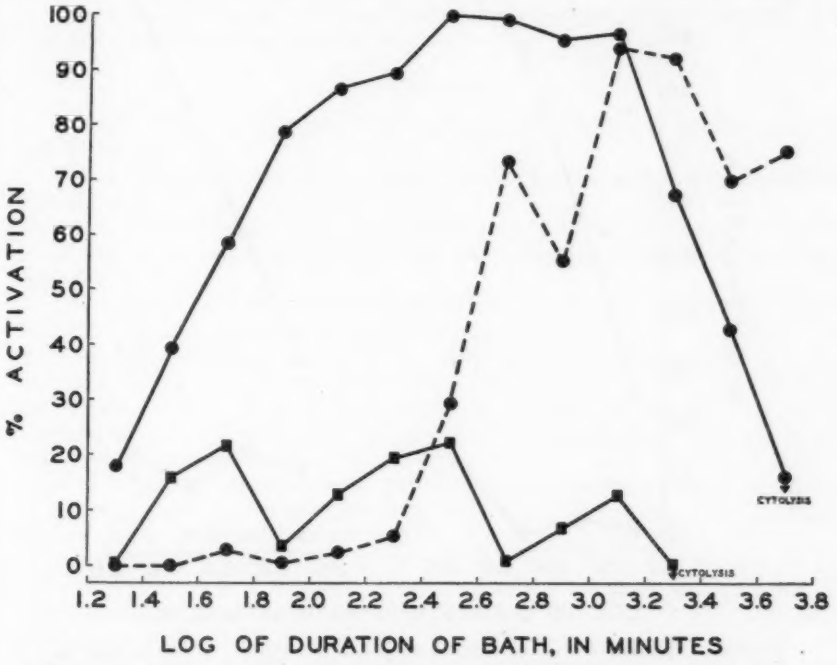

FIGURE 1. Relation of previous baths in picric acid to heat-activation of *Nereis* eggs.

Solid line connecting circular points—eggs heated after bath in M/1000 picric acid in sea water.

Solid line connecting square points—eggs heated after bath in sea water.

Broken line—eggs removed, unheated, from bath in M/1000 picric acid in sea water.

Each point is the average of all experiments performed in the logarithmic time interval denoted at the base-line. See text for further explanation.

from Figure 1 that the unfertilized eggs survived in the acid about twice as long as in sea water. Removal from sea water to the acid just prior to the expected onset of cytolysis (about 30 hours after removal from the animal) preserved the eggs as well as, but no better than, storage in the acid from the beginning.

KCl mixtures—The synergistic action of KCl mixtures and removal from picric acid to sea water was tested in 14 experiments, summarized in Figure 2(a). After 2–8 hours in the acid solutions, samples of eggs were removed to sea water and

to 5 per cent KCl mixtures; a control sample of the same batch of eggs kept in sea water was simultaneously exposed to the 5 per cent KCl mixture. This concentration of KCl is just below that necessary to induce regularly an appreciable percentage of response in ordinary eggs. Though the combined treatment was not in every case sufficient to activate the eggs, most experiments showed a pronounced synergism, and none showed a difference in the opposite direction. The response

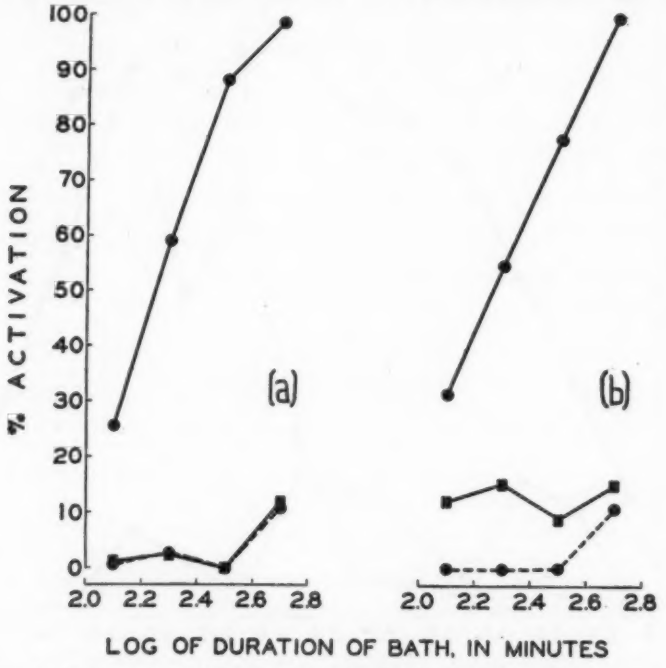

FIGURE 2. Relation of previous baths in picric acid to activation of *Nereis* eggs by (a) KCl mixtures, (b) sodium citrate mixtures.

Solid line connecting circular points—eggs treated after bath in M/1000 picric acid in sea water.

Solid line connecting square points—eggs treated after bath in sea water.

Broken line—eggs removed, untreated, from bath in M/1000 picric acid in sea water.

Each point is the average of all experiments performed in the logarithmic time interval denoted at the base-line. See text for further explanation.

of the eggs in these experiments, upon removal from picric acid baths to sea water, was somewhat less than average; thus the broken lines in the graphs in Figure 2 differ somewhat from the similar curve in Figure 1.

Sodium citrate mixtures—The same action was demonstrated with 10 per cent sodium citrate in place of the 5 per cent KCl mixture; 14 of 19 experiments showed a decided synergism. Five failed to show any significant difference between control and experimental. These failures were all among the shorter expo-

sures to the acid; the longer baths always resulted in increased sensitivity of the eggs to the citrate. This is illustrated clearly in Figure 2(b), which includes the data from all 19 experiments.

Ultra-violet irradiation—All attempts to show synergism between ultra-violet irradiation and removal from picric acid baths failed. A Uviarc mercury-vapor lamp, operating at 110 volts, 60 cycles, was used. The intensity of the radiation at the point at which the eggs were exposed was on the order of 6000 micro-watts per square centimeter.[3] Under such conditions, no significant differences could be found between the response to irradiation of eggs just removed from picric acid baths and those from sea water.

Discussion

The data illustrate three aspects of the action of picric acid in relation to activation of the eggs:

(1) in the presence of certain concentrations of picric acid, heat-activation and chemical activation are prevented;

(2) removal from the same concentrations of picric acid to sea water, after a short stay in the acid, acts synergistically with other activating agents in causing nuclear breakdown;

(3) removal from the acid to sea water after longer stays in the acid leads to activation without assistance from other agents.

That fertilization and maturation of marine eggs is inhibited by acids is a common observation (Clowes and Greisheimer, 1920; Smith and Clowes, 1924; Tyler and Schultz, 1932; Tyler and Scheer, 1937); so that the inhibition by picric acid of artificial activation is not surprising. Similarly, the preservation of the unfertilized egg in picric acid against cytolysis and death is in accordance with many observations of this action of acids; some treatments were reported far more effective in this respect than picric acid appeared to be (Carter, 1931; Just, 1920; Smith and Clowes, 1924; Tyler and Horowitz, 1937a; Tyler and Dessel, 1939). The suggestion has even been made (Tyler, Ricci, and Horowitz, 1938) that the greater life-span of eggs in alcohol, dextrose, anoxic media, etc. (Gorham and Tower, 1902; Loeb, 1902; Loeb and Lewis, 1902; Lillie, 1931; Whitaker, 1937), can be explained in each case by the production of acids. The only odd aspect of the action of picric acid in this regard is that eggs stored in it for some time are subsequently over-sensitive to stimulators, and eventually are activated merely by removal to sea water. This was observed after a stay in the acid of as much as 70 hours. This is entirely dissimilar to the acid activation of starfish eggs, as investigated extensively by Lillie (1926, 1927, 1934, 1941). Lillie's exposures were of only a few minutes' duration, and the eggs were visibly altered while in the acid; a slightly prolonged exposure destroyed the eggs altogether. In picric acid, however, the eggs remain apparently unchanged for days, but immediately respond when removed to sea water.

This fact leads to the postulate that picric acid may react with, or in some way inactivate, an activating agent produced within the egg. Above a certain concentration, this agent would lead to activation of the egg; in still greater concentration, or under other conditions, to cytolysis. This agent is apparently being constantly

[3] Thanks are due to Dr. A. C. Giese for this measurement.

produced, and either diffuses from the egg, or is gradually destroyed as it is produced. But when picric acid is present within the egg, this agent is retained by the acid in an inactive form; when the egg is removed to sea water, the picric acid diffuses away, in turn releasing any acid bound with the activating agent. Thus the inhibition is removed, so that there is a sudden release of the accumulated activator within the egg, causing a response if the accumulation has been great enough.

Such a suggestion is in harmony with the synergism found between other activating agents and the removal from picric acid after exposures of lesser duration, and with the temporal pattern of the development of this synergism, as shown in Figures 1 and 2. The activating agents may be supposed to act by accelerating the production of the hypothetical activating substance; subliminal doses of these agents may then produce enough of the substance so that the added quantity released from the picric acid suffices to produce the response. That a still greater concentration may lead to cytolysis is indicated by the fact that less activation, with considerable cytolysis, is found when eggs are heated after a very prolonged exposure to picric acid, than when they are simply removed from the acid at the same time to sea water, without heating (Figure 1).

TABLE II

Synergism between various activating agents in stimulation of Nereis eggs

Activating agents		No. of expts.	Per cent activation		
A	B		A alone	B alone	Both
Heat	Sodium citrate 5%	9	24	0	56
	10%	13	20	4	83
KCl 5%	Sodium citrate 6%	1	0	0	86
	8%	1	0	2	99
	10%	1	0	78	100
Heat, without usual stirring	Stirring	4	15	0	39

The synergistic action indicates that at least to some extent activation is brought about through the same channels by all four agents: heat, KCl, sodium citrate, and removal from picric acid to ordinary sea water. Added evidence in this direction was obtained in experiments showing pronounced synergistic action between heat and citrate mixtures, and between KCl mixtures and citrate mixtures (Table II). As previously mentioned, stirring during exposure to heat had a pronounced enhancing action on the stimulatory effect of the heat, but stirring did not appear to act similarly in connection with the chemical activators. Mathews (1901) reported that Loeb and Fischer had been able to activate *Nereis* eggs by mechanical agitation alone, but all attempts in this direction failed.

Attempts to show synergism between ultra-violet irradiation and sodium citrate mixtures or removal from picric acid all failed; this is in keeping with the failure of picric acid to inhibit activation by ultra-violet rays. This may indicate that the radiation acts through a different mechanism than that involved in stimulation with the other agents. But under the conditions of the experiments the duration of

the exposures to ultra-violet was on the order of 30–60 seconds, much less than with the other types of activation; this difference in the rate of activation may be the entire explanation for the non-conformance of the experiments with this type of activation.

Heilbrunn (1925), Heilbrunn and Wilbur (1937), and Wilbur (1939, 1941) have presented several lines of evidence indicating that the breakdown of the germinal vesicle in the *Nereis* egg involves a reaction of calcium ions with the colloids of the protoplasm, and an associated set of changes in viscosity. Heilbrunn proposed that stimulating agents act by freeing calcium ions from combination (with lipoprotein) in the cell cortex, so that the calcium may react with the inner protoplasm; this interpretation of stimulation has been applied not only to the eggs of *Nereis,* but to cells in general. If such a mechanism is actually involved in the response of the *Nereis* egg, it might be expected that a penetrating acid would inhibit activation. The picric acid might acidify the protoplasm to the extent that the amphoteric protein molecules would become predominantly cations, with less Ca-binding capacity than previously. This interpretation would perhaps also explain the activation found upon removal of eggs from picric acid baths to sea water; the calcium freed from the cortex by the acid could react with the cell interior upon removal of the acid. Thus the same agent would act, in a sense, both as activator and as anesthetic. A serious difficulty with this explanation of the data lies in the fact that the eggs must be left in the acid for several hours, if they are to respond upon removal to sea water. This would require the assumption that the liberation by the acid of calcium ions from the cortex is a very slow process; or else that the acid continues to accumulate within the egg over a period of hours, quickly rising to the inhibitory concentration, but only after hours attaining the concentration active on the cortex. Neither of these assumptions is impossible, but both are rather involved.

If the action of picric acid were due to this proposed effect on the Ca-binding properties of proteins, other acids might be expected to act similarly. The action of other acids similar to picric, as regards pK and penetrating ability, has not yet been investigated; however, acetic, boric, and tannic acids have been used in experiments similar to those performed with picric acid. Acetic acid was used in concentrations from M/6000 to M/300; boric acid, from $M/10^5$ to M/5; and tannic acid, from $M/10^6$ to M/100; the upper limits of concentrations used were factors of the solubility and the effects of the acids on the eggs. Over M/1000, acetic acid often injured the eggs irreversibly, so that they were not fertilizable; this makes it difficult to evaluate cases of inhibition by acetic acid of activation, in the absence of tests for reversal of the effect. Ten to twenty experiments were performed with each acid in attempts to demonstrate synergism with heat, in the manner of picric acid; the duration of the baths ranged from 30 minutes to 24 hours. On a few occasions, acetic acid in concentrations around M/500 (concentrations not always innocuous) showed the synergistic action, but as often acted in the opposite manner (probably because of injury to the eggs). On one batch of eggs, M/10–M/20 boric acid also showed some synergistic action with heat, but this did not recur in similar experiments with other batches of eggs.

A further corroboration of the interpretation in terms of an activator-substance was sought in several attempts to accumulate the activator more rapidly by heating the eggs in picric acid, with subsequent release to sea water. In only 3 of 16 such

experiments was there markedly more activation in the eggs so treated than in those similarly exposed to the acid without application of the heat. However, none showed differences in the other direction; no data of any experiment thus far performed militates against the suggested scheme.

The completely reversible inhibitory action of picric acid is similar to the action of isotonic citrate in the experiments of Heilbrunn and Wilbur (1937) and Wilbur (1941). Since the citrate is presumed to act by removing calcium ions from solution by the formation of calcium citrate, there is a suggestion that perhaps calcium picrate is a similarly weakly dissociated salt. However, a few measurements of the electrical resistance of calcium picrate solutions showed that the equivalent conductance increased only slightly with dilution over the range n/100–n/10,000. (The increase was in proportion to that found with $CaCl_2$ in the same concentrations; the equivalent conductance of calcium citrate increased enormously with dilution over this range of concentration.) Thus the inhibitory action of picrate cannot be explained on the same basis as that applied to citrate inhibition.

In their extensive experiments on the peculiar action of many substituted phenols on the eggs of the sea-urchin, Clowes and Krahl (1936), Krahl and Clowes (1936, 1940), and Tyler and Horowitz (1937b, 1938) found picric acid one of only two or three inactive members of this group of compounds. Inhibition of cleavage was encountered only at concentrations around M/100 or more, and the stimulation to respiration characteristic of this chemical group was lacking altogether. The calculations of Tyler and Horowitz showed that the concentration of dissociated picrate inside the cells was about 100 × that at which the related substances showed similar effectiveness. The same sort of relation was found for the other relatively inactive phenols. The latter should be tested in experiments similar to those with picric acid reported here. Such investigations might aid in deciding whether the action of picric acid is to be attributed to its acidity or to its particular molecular configuration.

Perhaps picric acid is unique in its combination of a low pK and a rapid rate of penetration into cells. The other phenols found to be exceptional (as regards inhibition of cleavage and stimulation to respiration) may share this combination of properties. Additional experimentation involving alteration of the picrate/picric acid ratio in the solution (through addition of HCl or NaOH) is also suggested; such data might strongly indicate whether the acidity or the picrate itself is the active factor. On either basis, the present data clearly show that, while anesthetizing the eggs, this active factor constantly renders them increasingly sensitive to the removal of the anesthetization, and to subsequent stimuli. Proper interpretation of this fact might lead to a significant contribution to the understanding of the nature of stimulation.

Summary

1. Germinal vesicle breakdown in *Nereis limbata* eggs, brought about by heat, or addition of KCl or sodium citrate to the sea water, was inhibited by the addition of picric acid at about M/1000.

2. After immersion for a few hours in M/1000 picric acid in sea water, germinal vesicle breakdown occurred upon application of subliminal doses of heat, KCl, or sodium citrate.

3. After immersion for 6–70 hours, removal of the eggs from picric acid to ordinary sea water caused germinal vesicle breakdown.

4. Activation by ultra-violet irradiation did not conform in these relations to picric acid, under the conditions of the experiments.

5. These results are interpreted on the basis of a hypothetical activating substance produced within the egg, and inactivated or bound by picric acid.

6. The relation of picric acid to the calcium ion and the combination of calcium with protoplasmic proteins is considered, in an alternative explanation of the results.

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A STUDY OF THE GOLGI APPARATUS IN CHICKEN GIZZARD EPITHELIUM BY MEANS OF THE QUARTZ MICROSCOPE

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The only fact about the Golgi apparatus that is universally accepted is that it is a cytoplasmic constituent of most cells which, after special fixation, blackens with silver nitrate or osmic acid. Controversies as to its structure, composition, function, and even its existence in the living cell, have been carried on continuously ever since Golgi originally described such a cellular constituent in 1898, totaling considerably over 2000 published papers. Mere descriptions of the blackened apparatus appear to be no longer fruitful. Furthermore, since cells must differ in order to carry on their specialized functions, warnings have been voiced against making hasty generalizations about all cells from studies on particular cells. The work reported in this paper pretends to nothing further than an analysis of certain features in one type of cell.

The Golgi apparatus in the lining of the chicken gizzard near its junction with the proventriculus is unusually spectacular and easily demonstrated. It can practically always be clearly shown after the usual osmic acid or silver nitrate techniques. Moreover, its size is enormous (Fig. 5). For these reasons chicken gizzard material is excellent for the study of the nature of the peculiar region of the cytoplasm where this network appears.

Previous studies have followed its changes in form during embryonic development of the gizzard (Hibbard, 1942). It can be demonstrated only in fixed material after appropriate impregnations. It can never be seen in living cells or in cells otherwise well fixed but not fixed by the usual methods for the Golgi apparatus. The only cytoplasmic inclusions which are rendered visible by methods other than silver and osmic impregnations in the general zone occupied by the Golgi apparatus, are vacuoles which may be stained vitally or postvitally with neutral red, and occasional filamentous mitocondria. Hibbard (1942) has suggested that these vacuoles might be the antecedents of the typical Golgi network.

In an interesting series of papers, Worley (1943, 1944) has pointed out the high susceptibility of cytoplasmic inclusions, in many types of cells, to displacement or to changes in form and appearance with very slight changes in salt concentration in the surrounding fluid. Within ten seconds such distortions may take place so that quite different bodies from the original ones may be formed. Worley suggests, as Parat did nearly twenty years ago, that the fixed picture as it appears in sections may not at all resemble the living conditions.

Analysis of the Golgi apparatus by special types of illumination is not new. Monné in 1939 published two papers dealing with the appearance of the Golgi apparatus in Helix spermatocytes, using polarized light in one case and dark field illumination in the other, in order to demonstrate physical characteristics of difference

between the Golgi bodies and other cytoplasmic constituents. It must be remembered that his results apply to spermatocytes only and great care should be taken not to generalize them to apply to all Golgi bodies unless similar observations are made on other types of cells. There is a great difference between the spermatocyte Golgi apparatus and that in the glandular cells described in this paper, in staining reactions, susceptibility to deformation and the variety of methods by which it may be seen at all (Hibbard, 1945).

The present study was undertaken to determine, by means of ultraviolet microphotography, something about the nature of the cytoplasmic zone which becomes a complex network of blackened material after impregnation. It is not a study of the living cell, and may therefore be the analysis of an artifact. But if so it is one of extremely uniform occurrence in the chicken gizzard and one which undoubtedly has some precursor substance or some physical state of the material in the living cell which produces the localized black network in the fixed sections in perfectly regular fashion.

The technique employed was to make 5 μ sections, either in paraffin or frozen, of material fixed in 7 per cent neutral formalin or in Da Fano, one of the fixatives nearly always successful in demonstrating the Golgi apparatus in gizzard epithelium. These sections were mounted on quartz slides under quartz coverslips and photographed with the quartz microscope using the 2537 Å line of mercury as the light source. The sections were unstained. Lavin (1943) has described the technique of ultraviolet microphotography.

Examination of the microphotographs shows that most cells have no peculiar tone variation in the zone in question (Fig. 3). However, in those cells with any apparent difference in the Golgi zone as compared with the rest of the cytoplasm, the Golgi zone appears somewhat paler (Figs. 1, 2, and 4).

It is known that nucleic acid has an absorption maximum of 2600 Å and proteins which contain tyrosine and tryptophane have an absorption maximum at 2800 Å. Nucleoproteins will have a maximum at some point intermediate. In order to show that these materials absorb in the ultraviolet region of the spectrum while in the solid state, microphotographs of globulin and of nucleic acid pellets were taken with the quartz microscope. They were cut and treated as if they were blocks of tissue. These photographs are reproduced in Figure 7 and Figure 8. It will be noted that while the nucleic acid is dark, the globulin remains pale. In a similar way microphotographs taken in the ultraviolet should demonstrate in tissues, with-

FIGURE 1. Microphotograph, ultraviolet illumination; 5 μ paraffin section after Da Fano fixation; no stain.

FIGURE 2. Microphotograph, ultraviolet illumination; 5 μ paraffin section after 7 per cent formalin fixation; no stain.

FIGURE 3. Microphotograph, ultraviolet illumination; 5 μ frozen section after 7 per cent formalin fixation; no stain.

FIGURE 4. Microphotograph, ultraviolet illumination; 5 μ paraffin section after 7 per cent formalin fixation; no stain.

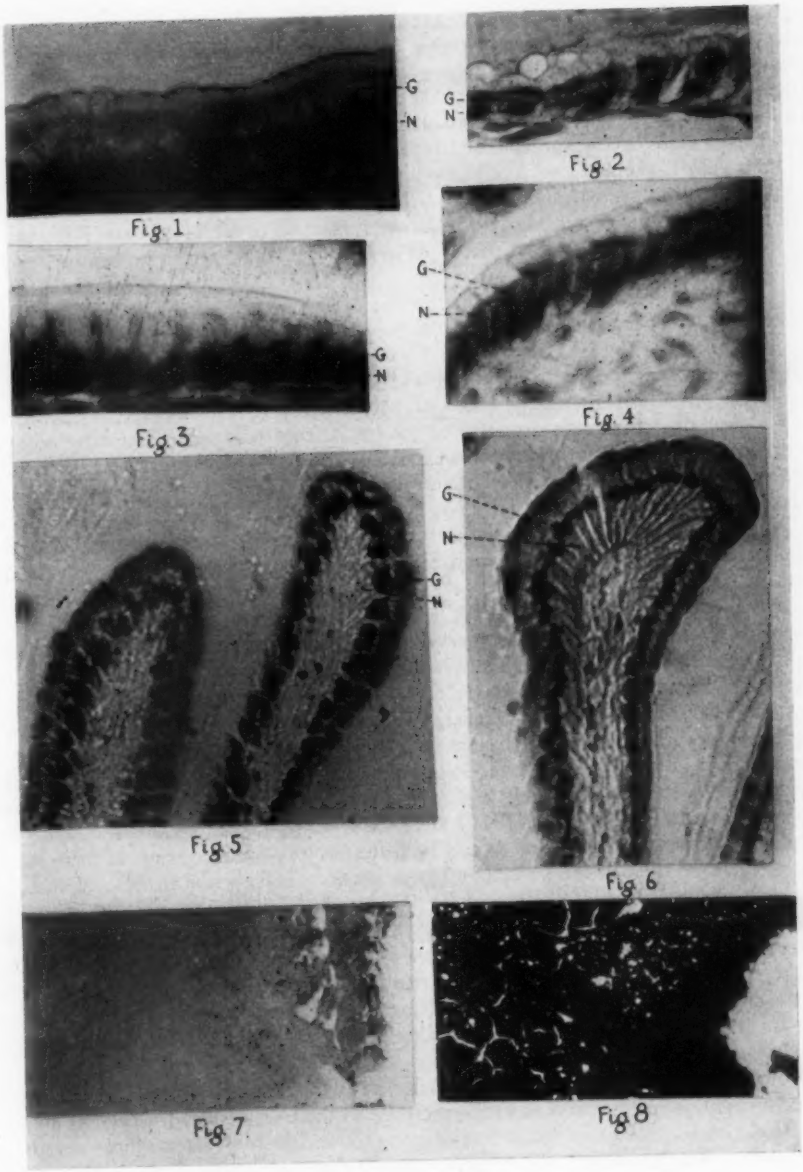
FIGURE 5. Microphotograph, visible light with green filter; 5 μ paraffin section after Da Fano fixation followed by silver nitrate impregnation and reduction; no further stain.

FIGURE 6. Same as Figure 5.

FIGURE 7. Microphotograph, ultraviolet illumination; section of a pellet of globulin.

FIGURE 8. Microphotograph, ultraviolet illumination; section of a pellet of nucleic acid.

(In all figures, G—Golgi zone and N—nuclear region.)



FIGURES 1–8

out the necessity of staining, the presence of any substance of appropriate absorption maximum. Since the absorptive capacities of many organic tissue components are known and thus this method of demonstrating cell inclusions is a reflection of their chemical composition, we may say that the photographs present evidence that the Golgi zone does not contain appreciable amounts of nucleoproteins, nucleic acids or proteins containing tryptophane and tyrosine. The position of the Golgi apparatus in most cells is characteristically in close proximity to the nuclear surface, a position of possible physiological importance. This evidence that the region does not contain nucleoproteins or nucleic acid in large amounts if at all, is of some importance, particularly in view of the fact that most histological stains are in no sense chemical tests.

Our results also shed some light on the further question, which is of some interest: why does special fixation have to be practised before the subsequent impregnation will "take?" Examination of Figures 1, 2, and 4 will show that the Golgi zone is paler than the rest of the cytoplasm in many of the cells. In all probability this clear zone corresponds to the area blackened by silver nitrate. The curious fact is that this paler area may appear after formalin fixation whether the sections are imbedded in paraffin or frozen (Figures 2, 3, and 4), and also after Da Fano fixation without subsequent silver impregnation (Fig. 1). Silver impregnation applied after Da Fano fixation will produce a spectacular type of Golgi apparatus as shown in Figures 5 and 6, while exactly similar impregnation after neutral formalin as the fixative will produce only miscellaneous black granules throughout the cell with no greater blackening of the Golgi zone than of other regions. This seems to indicate that the Da Fano fixative either preserves some constituent lost in the formalin, or else adds itself to material already there, to make it reduce the silver in the conspicuous network. The identical absorption capacities of the region to ultraviolet light, whether the fixative be formalin or Da Fano, suggests a similarity in the quality of the fixed protoplasm. Why the Da Fano fixative should create a focal point exactly in the Golgi zone for the subsequent reduction of the silver, is not so clear. It may be that the Golgi zone is the site of aqueous vacuoles in the living cell, possibly containing highly dispersed materials such as proteins and lipoids, as found by Simpson (1941) after the freezing-drying technique. Both the work of Simpson and our own would indicate far less concentration of proteins in the Golgi zone than in the surrounding cytoplasm. During the process of fixation there may be a distortion of the zone as Parat thought, and more recently, Worley; and the distorted "apparatus" may be fixed by any fixative that coagulates the cytoplasm around it. The similar appearance of the apparatus as shown by ultraviolet photography, whether the fixative be formalin or Da Fano, shows that the reduction of the silver on or in the apparatus subsequent to fixation depends, in all probability, not on the fidelity of the whole cell's fixation but on the character of the fixative used.

In conclusion, these studies of cells by means of ultraviolet photography give certain negative information as to the material in the Golgi zone: it does not appear that it is nucleoprotein or nucleic acid, except possibly in greater dilution than in the rest of the cytoplasm. They also suggest that successful silver impregnation after one fixative and not after another may be due, not to less faithful fixation of the cell, but to a more direct relation between the fixative and the silver. Finally it

must be remembered that these studies were made exclusively on fixed material and there is much evidence that the morphology of the cellular constituents in such material does not coincide with that in living cells.

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DILUTION MEDIUM AND SURVIVAL OF THE SPERMATOZOA OF ARBACIA PUNCTULATA.* I. EFFECT OF THE MEDIUM ON FERTILIZING POWER

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Introduction

The investigations in sperm physiology may be roughly divided into two principal aspects. First, there is the problem of the role of the sperm cell in fertilization. Second, there is the problem of the survival of spermatozoa as a fundamental condition for the survival of the species. These two aspects each have their own long lists of investigations.

The study of the sperm cell in fertilization has produced one outstanding theory. This is the Fertilizin Theory of Lillie (1914). The course of investigations, past and recent, shows that this theory, although not completely confirmed and probably in need of modification, has been found useful by many workers in the field. According to the theory, the male germ cell is the carrier of a substance, the "sperm receptor," which is functional in the fertilization process. This substance is thought to combine with "fertilizin," an egg secretion. The complex of sperm-receptor-fertilizin then reacts with an "egg receptor" to form a three-way complex in the egg. The formation of this ternary complex initiates the fertilization reactions of the egg.

The study of sperm senescence, in contrast to the above, has yielded results which are, at best, unsatisfactory. Gray (1928a and b), who investigated the changes in metabolism of sperm under various conditions, reported that sperm in highly concentrated condition have a very low rate of respiration. If diluted, the sperm show a burst of metabolic activity. The greater the dilution, the more intense is this burst of action, although of shorter duration. Gray advanced the hypothesis that a large part of the sperm cell's internal supply of fuel was used up in the first burst of energy, so that the greater its intensity, proportionately shorter became the life of the spermatozoön. The initial burst of activity was in turn determined by the available "free space" in which the sperm cell could move, that is, by the dilution. In the limited space available to each cell in the concentrated suspensions, the sperm cell was only incompletely activated, and, hence, its life was prolonged.

This explanation cannot be applied without certain limitations. If it were, a single spermatozoön placed in an infinitely large volume of diluent would end its metabolism instantly. Further, "mechanical crowding" as an explanation is applicable only to the translatory or vibratory activity of the sperm and not to the respiratory activity. With all the known variables, such as oxygen and carbon dioxide tensions, rigidly controlled, Gray's evidence shows that when "free space" is available the rate of sperm respiration increases. "Mechanical crowding" is thus

* Work done as part of the requirement for the degree of Doctor of Philosophy.

not an explanation for the changes in respiratory rate but a description of the conditions under which the respiratory rate is low. That is, it is logical to state that sperm are quiescent because they are forced to be immobile, but it is not logical to state that sperm respire at a high rate because they are no longer forced to be immobile. Such a statement has implications of teleology. There must exist an unknown factor which, under conditions of dilution, brings about the increased respiration of sperm. Undiluted sperm, therefore, must be a system composed of the cells plus the unknown factor. Dilution of the system, not the dilution of cells alone, brings about the respiratory activity of the spermatozoa.

The foregoing review shows that Lillie's fertilization studies have indicated the existence of a substance that determines the fertilizing power of the sperm cell. The review shows, too, that Gray's work has neglected one variable, the sperm cell medium, or a factor in that medium which affects the duration of metabolic activity of the sperm cell. It is the purpose of this research to present evidence for the existence of a single factor that influences the conservation of fertilizing power by sperm and the respiratory activity of sperm. The work is presented in two sections, the first section dealing with the fertilizing capacity of sperm, and the second with the respiratory activity of sperm.

The author is greatly indebted to Dr. Daniel Mazia for his guidance and helpful suggestions.

Materials and Methods

The materials used in the series of experiments to be described were the germ cells of the Atlantic sea-urchin, *Arbacia punctulata*. The general methods and precautions outlined by Just (1939) were followed carefully. To obtain the germ cells, the urchins were thoroughly washed in running sea water and running tap-water, after which they were dried carefully with clean cheese-cloth. A cut around the oral region disclosed the sex of the animal. If male, the sperm exuding from the genital pores were received in a dry stender dish; if female, the animal was allowed to shed the eggs into a stender dish filled with sea water.

The sperm suspensions for the earlier experiments were made according to the "drop" method of Lillie (1913). For greater precision in later experiments, sperm were "packed" by centrifugation at 3500 r.p.m. for 30 minutes. These packed sperm cells were drawn into a calibrated capillary tube. The tip of the capillary was wiped clean, and the contents were used to make the sperm suspension. The capillary was calibrated by taking up the same volume of re-distilled mercury and weighing the mercury accurately.

As a check on the constancy of this method, sperm counts were made. A unit quantity of packed sperm was suspended in one cc. of sea water, shaken thoroughly, and 0.01 cc. of Bouin's fixative added. This suspension was diluted one hundred times, and the number of sperm present counted in a haemocytometer chamber. The results are given in Table I, and it was found that the greatest deviation from the average was in the order of 6 per cent, a constancy not attainable by the "drop" method.

The seminal fluid used in the experiments was collected simply by drawing off the supernatant fluid from the packed "dry" sperm after centrifugation.

The egg suspensions were made by washing the eggs several times in sea water and allowing them to settle in the dish by force of gravity. Equal samples of the

TABLE I

Sperm count, using 0.00155 cc. packed sperm per cc. of sea water, diluted 100 times

Suspension	Number squares counted	Total counted	Average number per sq.	Conc. of packed sperm per cc.
No. 1	32	386	12.0	3.06×10^{12}
No. 2	32	346	10.8	2.76×10^{12}
No. 3	32	379	11.8	3.02×10^{12}
No. 4	32	360	11.2	2.86×10^{12}
No. 5	32	390	12.1	3.09×10^{12}
No. 6	32	359	11.2	2.86×10^{12}
Average				2.94×10^{12}

settled eggs were diluted in varying amounts of sea water, mixed to give homogeneity, and aliquots were removed with a calibrated pipette. From the number of eggs present per unit length, the total number of eggs could be calculated. One drop of a suspension of suitable egg-concentration was placed in 5 cc. of sea water. Generally, the number of eggs in one insemination test was 750–1000. As Lillie (1915a) had shown, variations of this order in the total number of eggs used in the inseminations do not affect the final results appreciably. Since a fresh egg suspension was used for the insemination tests at any one time, the tests at two different times used different suspensions whose concentrations varied somewhat, so that the results were possibly not comparable. Those tests run at any one time used the same egg suspension, and, therefore, the results were comparable to each other.

For the insemination, a unit quantity of the sperm suspension in a pipette was carefully squeezed out over the eggs, and the whole dish gently and uniformly stirred. For determination of fertilizing power, the percentage of eggs activated was calculated by counting a minimum of 200 eggs.

Widely diverging types of experiments were made in the course of this investigation, each type entailing its own methods and techniques. Because of this, other methods and techniques will be described in connection with particular experiments. Each typical experiment to be presented in the following section was one of a minimum of five experiments all giving similar results.

EXPERIMENTS AND RESULTS

The seminal fluid factor and the survival of sperm

Past researches have shown that sperm in the undiluted condition freshly-exuded from the testes are immobile, and that the sperm manifest intense activity upon dilution with sea water. Subsequently, the fertilizing power of the sperm cells declines sharply and within a relatively short time. Workers in the past had diluted sperm fresh from the testes with sea water. Since the medium seemed to be a variable in this type of dilution, and since sperm cells in the testes were suspended in a liquid medium, a factor influencing the fertilizing capacity of sperm cells was sought in the seminal fluid.

To examine the effect of the seminal fluid on the fertilizing power of sperm, a series of experiments was done using sperm suspensions of the same concentration in seminal fluid and in sea water. These suspensions were then tested at different time intervals for their fertilizing power. In the experiment shown in Table II, a 0.4 per cent sperm suspension (according to the terminology of Lillie) was used. One drop of the suspension was used to inseminate 750–1000 eggs. The formation of the fertilization membrane was used as the index of activation of the egg.

The effect of the seminal fluid in promoting the survival of the sperm was apparent even after five hours, and after 12 hours, when the sperm in sea water were completely non-functional, a large number of those in the seminal fluid were still capable of bringing about activation. At each test, microscopic observation revealed that the per cent activation of eggs was approximately directly proportional to the number of motile sperm.

TABLE II

Activation of eggs by sperm suspensions of 0.4 per cent concentration in seminal fluid and sea water

Medium	Per cent activation					
	10 a.m.	11 a.m.	3 p.m.	5 p.m.	8 p.m.	10 p.m.
Sea water	100	100	46	22	0–2	0
Sem. fluid	100	100	99	100	99	95

The maintenance of fertilizing power of the sperm cells was a function specific for the seminal fluid. Experiments were made using the perivisceral fluid as the suspension medium. The perivisceral fluid was found to have a toxic effect on the retention of fertilizing power by sperm.

It seemed clear that in the seminal fluid an unknown factor was enabling the sperm to retain their fertilizing power for a long period of time. In view of the work of Cohn (1918), a check of the effect of pH became necessary. The pH of the seminal fluid was measured electrometrically with McGinnis' electrode. A number of such measurements showed the pH of seminal fluid to vary between 7.6 and 7.9.[1] Experiments were done comparing the survival of sperm in seminal fluid and sea water acidified to the same pH as the seminal fluid sample.

In the same experiments, another chemical property of the seminal fluid was investigated, namely, the heat-sensitivity. A sample of the seminal fluid in a test tube was heated at 100° C. for ten minutes, the seminal fluid allowed to cool to room temperature, and this heated seminal fluid was tested for its effect on the survival of sperm.

The results of experiments are summarized in Table III. The dilution used was one drop of centrifuged sperm to 5 cc. of medium. The pH of this seminal fluid sample was 7.72; the sea water (pH 8.0) was acidified to 7.7 by the addition of 11 drops of 0.1 N HCl to 100 cc. of sea water. All the suspensions were made at 5 p.m.

The results showed that acid sea water maintained the fertilizing power of the sperm only slightly longer than normal sea water and not nearly so long as the

[1] Done by Mr. M. E. Smith, of the MBL staff.

TABLE III

The effects of pH, heated seminal fluid on the survival of sperm, as shown by time measurements of the fertilizing power

Medium	Per cent activation				
	5 p.m.	9 p.m.	10:30 p.m.	4 p.m.	10 p.m.
Sea water	100	100	14	0	0
Sem. fluid	100	100	100	97	73
Heated fluid	100	0	0	0	0
Acid s.w.	100	100	40	2	0

seminal fluid. The heated seminal fluid, on the other hand, had clearly lost the function of promoting the survival of the sperm cells. It was evident that pH was not the effective factor in the seminal fluid and that the effective factor was heat-sensitive.

This heat sensitivity led to the suspicion that the unknown factor was protein. To test this hypothesis, the seminal fluid was saturated with ammonium sulfate. A faintly rose-colored precipitate resulted from this treatment. This precipitate was filtered off, and the residue on the filter paper dissolved in a volume of sea water equal to the original volume of seminal fluid. The sea water containing the residue was then dialyzed against fresh changes of sea water in the refrigerator for 30 hours. The dialyzing membrane was commercial sausage skin (Cenco). This treatment removed the ammonium sulfate. The liquid inside the dialysis bag, essentially an artificial seminal fluid, was then used as the suspending medium for the sperm.

As controls for this experiment, various other media were used to suspend equal concentrations of the same sperm sample. For the first of these, the filtrate of the seminal fluid (seminal fluid minus the precipitated material) was also dialyzed against sea water for the same length of time as the residue solution, and this "dialyzed filtrate" was used as a suspending medium for the sperm. Normal sea water, acid sea water, and natural seminal fluid were also run as controls. The dilution used was one drop of centrifuged sperm to 10 cc. of medium, and the pH was carefully checked in each case.

The results (Table IV) showed that spermatozoa in the "artificial seminal fluid" retained their fertilizing power nine hours longer than did the sperm in sea water. From the data, it was concluded that the seminal fluid factor was precipitable with ammonium sulfate and non-dialyzable. The earlier conclusion as to the negligible effect of pH was confirmed in this experiment.

The idea of the seminal fluid factor's being protein seemed to be borne out and warranted an analysis of the seminal fluid for its protein content, along with determinations of other physical and chemical properties. For determination of protein, Folin's micro-Kjeldahl with direct Nesslerization was used, the solutions being compared in a photoelectric colorimeter. The results showed 2.5 mg. protein per cc. of 100 per cent seminal fluid. The pH of the seminal fluid was found to vary between 7.6 and 7.9 as compared to the pH of sea water, which varied from 7.9 to 8.1.[2] The freezing point of seminal fluid was $-1.715°$ C. as compared to that of

[2] Done by Mr. M. E. Smith, of the MBL staff.

TABLE IV

The effects of various media on the survival of sperm, as shown by insemination tests

Medium	pH	Per cent activation					
		0.5 hrs.	5.5 hrs.	10.0 hrs.	15.5 hrs.	24.0 hrs.	26.0 hrs.
Sea water	8.0	98	92	49	13	0	0
Acid sea water	7.7	98	87	72	68	0	0
Sem. fluid	7.6	100	97	100	100	98	35
Dial. residue	7.8	99	96	98	85	19	4
Dial. filtrate	7.8	93	80	20	5	0	0
Eggs tested		98	98	100	100	98	100

sea water, which was $-1.892°$ C.[3] Chloride analysis showed the sea water to contain 0.508 moles per liter, while the seminal fluid contained 0.590 moles per liter.[4] Analysis for glucose (reducing sugar) showed the seminal fluid to contain less than 10 gamma in 5 cc.

These results suggested as one possibility that the action of seminal fluid on the sperm could be attributed to the osmotic pressure difference between the seminal fluid and the sea water. The demonstrated heat-sensitivity of the seminal fluid factor, however, ruled this possibility as unlikely, as did the prolonged dialysis of the last experiment given, for such treatment would equalize the osmotic pressure of the seminal fluid with that of the sea water.

The difference in chloride content between the seminal fluid and sea water was not considered as a factor in prolonging the fertilizing power of the sperm cells. The prolonged dialysis described earlier would have equalized the chloride concentration of the sea water and the "artificial seminal fluid" of Table IV, yet these two media had markedly different effects upon the sperm cells. Also, the demonstrated heat sensitivity of the seminal fluid factor indicated that it was not chloride.

The effective seminal fluid factor therefore seemed to be protein, but protein, by its presence, would establish a colloidal osmotic pressure which might be the agency acting on the sperm.

In Table IV, it may be noted that the "dialyzed residue" was not as effective as the natural seminal fluid. There are several possible explanations. First, during the prolonged dialysis, some of the protein may have been denatured, a point to be checked in future investigations. Second, the concentration of the factor in the "artificial seminal fluid" was probably not equal to that in the natural medium, due to some loss of protein in handling, and difficulties in volume control in dialysis.

At this point, attention should be called to the fact that still another possibility existed as to the manner in which the seminal fluid functions. This was the question of nutrition of the sperm by the seminal fluid. This question will, however, be taken up in the discussion.

There remained one mode of action of the seminal fluid factor hitherto uninvestigated. The results of the experiments already described validated the as-

[3] Done by Dr. Jay A. Smith, of the MBL staff.
[4] Done by Mr. J. Weissiger, of the MBL staff.

sumption that the seminal fluid factor acted in some manner upon the surface of the sperm cells.

Observations made during attempts to measure sperm activity in a capillary tube showed spermatozoa to be positively thigmotropic to glass surfaces. At the instant of contact, the spermatozoön lost a large part of its activity and rotated slowly about its point of contact. The observation seemed to show the presence of a surface active substance on the head of the spermatozoön. This fact, previously observed by Buller (1902), led to the following experiment.

Three suspensions of sperm of equal concentration were made in sea water. Suspension No. 1 was left untreated. Glass powder was added to suspensions No. 2 and No. 3. All three suspensions were shaken simultaneously and placed in the refrigerator, where the powdered glass was allowed to settle for three hours. Insemination tests were run to determine the relative sperm populations in these three suspensions. Qualitative microscopic observations on sperm population were also made at each dilution of the original suspensions as a check.

As shown in Table V, the results indicated that the sperm population in the second and third suspensions was greatly reduced, a result confirmed by microscopic observation. It was possible that the glass powder injured a large part of the total sperm population, but the absence of significant numbers of injured sperm seemed to indicate that the glass powder removed the missing sperm by adhesion.

TABLE V

Activation of eggs by progressive dilutions of sperm suspensions treated with glass powder as compared to untreated sperm suspension

Suspension	Undiluted	1:1 Dilution	3:1 Dilution
No. 1	100	100	100
No. 2	96	61	27
No. 3	100	75	35

A similar experiment was made to test the surface activity of seminal fluid protein, since the proposed surface-action implied the identity of sperm-surface-substance and seminal fluid protein. A sample of seminal fluid was divided into three portions. Portion No. 1 was left as the untreated control. Glass powder was added to portion No. 2, the portion shaken thoroughly, and the glass powder filtered off with Whatman No. 5 filter paper. Portion No. 3 was shaken three times, each time with fresh glass powder and filtered free of glass each time. These seminal fluid portions were then used to make sperm suspensions of equal concentration and tested for the maintenance of the fertilizing power. The results are given in Table VI.

Clearly, the glass powder removed the sperm-longevity factor from the seminal fluid, so that seminal fluid protein, too, seemed to be surface-active on glass. Although the experiments of Tables V and VI did not completely establish the identity of the seminal fluid factor and the substance on the surface of the sperm, they did show that both substances were apparently surface-active.

In furtherance of this line of thought, experiments were made to learn whether sperm in sea water gave off their surface substance into the surrounding medium.

TABLE VI

Removal of the factor from seminal fluid with glass powder

Medium	Per cent activation						
	0.0 hrs.	4.0 hrs.	9.5 hrs.	12.0 hrs.	14.5 hrs.	24.0 hrs.	28.0 hrs.
Sea water	100	100	78	65	40	0	0
Portion No. 1	100	100	100	100	100	100	95
Portion No. 2	100	100	100	80	54	5	0
Portion No. 3	100	99	1	0	0	0	0
Eggs tested	100	100	100	100	100	100	100

In one type of experiment, a heavy suspension of sperm in sea water was allowed to stand for several hours. The sperm were then removed by centrifugation and the supernatant fluid tested as a sperm medium. In another type of experiment, the above procedure was repeated several times, the supernatant fluid used to support a fresh sample of sperm after each centrifugation. After the final centrifugation, the supernatant fluid was tested for its effect on fresh sperm. In all cases, the results were negative. Such "sperm washings" had neither a detrimental nor favorable effect on the maintenance of the fertilizing power of the sperm.

There remained one other point of investigation in the survival time of spermatozoa. Observations had shown that seminal fluid protein, even in low concentration, was effective in maintaining spermatozoa. Gray (1928a) had postulated a "mechanical crowding" effect as the primary factor in the survival of sperm. Since he used "dry" sperm, which was composed of about 60 per cent seminal fluid, there arose the possibility that the longer survival of the more concentrated sperm had as its cause, not "mechanical crowding," but the larger amounts of seminal fluid protein carried over in the "dry" sperm. A test of this possibility followed.

A sperm suspension in seminal fluid was made by suspending 0.025 cc. of packed sperm in one cc. of seminal fluid. A second suspension was made by taking 0.2 cc. of the first suspension and adding it to another one cc. sample of seminal fluid. This serial dilution was repeated twice more, to make four sperm suspensions, all in seminal fluid. The operation was carried out quickly, the last suspension made within a minute of the first. The final concentrations of the four suspensions were, in Lillie's terminology, approximately 5 per cent, 1 per cent, 0.2 per cent, and 0.04 per cent, since packed sperm contained approximately twice the amount of sperm per unit volume as did the "dry" sperm used by Lillie. The insemination tests were made at the same dilution, each of the more concentrated suspensions being diluted to the lowest concentration of 0.04 per cent. One drop of this final suspension was used to inseminate the eggs. The results are given in Table VII.

A study of these results as compared to those of Gray showed that, even though Gray's results might be partly explained as the action of seminal fluid protein, "mechanical crowding" did seem to play a part in determining the life-span of the spermatozoa. However, it may be pointed out that this "crowding effect" seems to be non-linear in relation to the concentration, and is most apparent at extreme dilutions.

TABLE VII

The effect of concentration on the survival of sperm in seminal fluid

Suspension concentration	Per cent activation					
	0.5 hrs.	4.0 hrs.	7.5 hrs.	18.0 hrs.	23.0 hrs.	30.0 hrs.
5 per cent	88	90	78	77	85	91
1 per cent	81	85	83	79	83	72
0.2 per cent	90	92	84	80	49	13
0.04 per cent	92	95	86	45	10	0

The seminal fluid factor and its role in fertilization

In the course of the preceding experiments, sea water suspensions of sperm used to test the eggs showed a contrasting behavior as to fertilizing power. The individual spermatozoön in seminal fluid appeared to have a greater fertilizing power than the spermatozoön in sea water. An experiment was devised to investigate this more closely.

A volume of 0.025 cc. of packed sperm was suspended in one cc. of seminal fluid. Immediately after the suspension was made, one drop of the suspension was used to inseminate approximately 1000 eggs. Serial dilutions were made as for the previous experiment, but as each new suspension was made, one drop was used to inseminate approximately 1000 eggs. A sea water control was run, dilution and inseminations being made in the same way (Table VIII).

TABLE VIII

A comparison of the fertilizing power of sperm in seminal fluid and sperm in sea water

Medium	Per cent activation			
	1st dilution	2nd dilution	3rd dilution	4th dilution
Sea water	100	97	37	12
Sem. fluid	100	99	100	81

The results proved that there was a strong difference in the fertilizing power of the sperm cells in seminal fluid as compared to those cells in sea water. This difference became even more pronounced when the original concentrated suspensions were allowed to stand for ten hours, as shown in Table IX. Only the most concentrated suspensions in the seminal fluid and the sea water were kept. The dilutions were made anew.

The interpretations of these results were rather complex and will be discussed in a later section.[5]

The apparent increased fertilizing power of the sperm in the seminal fluid indicated that seminal fluid factor might be directly concerned with the fertilization process. It was recalled that Lillie (1915) had given as one of the criteria for the "sperm receptor" the power to "bind" agglutinin from the egg. Lillie meant by this

[5] See page 175.

TABLE IX

A comparison of the fertilizing power of sperm in seminal fluid and sea water after 10 hours

Medium	Per cent activation			
	1st dilution	2nd dilution	3rd dilution	4th dilution
Sea water	100	86	7	0
Sem. fluid	100	100	98	79

that if the agglutinin were treated with the "sperm receptor" solution (here the seminal fluid, presumably), the action of the agglutinin on the sperm would be greatly reduced. This experiment was done, with the expectation that, if the seminal fluid factor and the "sperm receptor" were one and the same, the agglutinating action of the egg secretion would be reduced.

A series of dry watch glasses was arranged. In the first, two drops of seminal fluid and two drops of egg-water were thoroughly mixed. Two drops of this mixture were then removed to the next watch glass and diluted with two drops of sea water. This treatment was repeated down the series. For the control, sea water was used instead of seminal fluid. For the test, a drop of a standard sperm suspension (0.00155 cc. packed sperm per cc. of sea water) was placed in the watch glass, out of contact with the mixture. The watch glass was then placed under the objective of the microscope, the two liquids (sperm suspension and sea water-seminal fluid mixture) shaken together, and the reaction of the sperm noted. In the following table, + indicates a positive agglutination, − a negative agglutination, and ± uncertain. The number of + symbols indicates the intensity of the reaction.

TABLE X

The agglutination reaction induced by dilutions of egg-water-seminal fluid mixtures, as compared to those induced by egg-water-sea water mixture of the same dilutions

Dilution	Sea water egg-water	Sem. fl. egg-water
1	+++	++++
1/2	+++	++++
1/4	+++	+++
1/8	++	+++
1/16	+	++
1/32	±	+
1/64	−	+
1/128	−	±

Instead of having its action on the sperm reduced, the results revealed that egg-water treated with seminal fluid had, if anything, a more powerful agglutinating power than the sea water-treated egg-water. In any event, the agglutinating power was not reduced. The only conclusion possible from these results seemed to be that the seminal fluid factor was not the "sperm receptor" of Lillie.

However, the data given indicated that the sperm reaction in the seminal fluid-egg-water mixture was more intense than the corresponding reaction of sperm in the egg-water sea water mixture. This phenomenon was put to a quantitative test.

Standard suspensions of sperm were made in seminal fluid and in sea water. The concentration was 0.00155 cc. packed sperm per cc. of medium. These suspensions were allowed to stand at room temperature (25° C.). At intervals, a drop from either one or the other of the suspensions was placed on a watch glass, out of contact of a mixture of one drop of egg-water and two drops of sea water. The liquids were shaken together under the microscope, and the time of agglutination (from onset to reversal) was taken with a stop-watch. The results are summarized in Table XI.

TABLE XI

The agglutination time of sperm suspended in seminal fluid as compared to that of sperm suspended in sea water

Time Tested	Agglutination time in seconds	
	Sperm in sea water	Sperm in seminal fluid
p.m.		
3:00	90	120
3:06	63	69
3:10	61	115
3:18	82	98
3:40	49	111
3:44	53	95
3:50	47	91
3:57	63	90
4:05	61	97
4:40	91	86
4:50	86	75
5:15	34	120
5:30	71	115
7:30	75	76

The data show that, on the average, the sperm in the seminal fluid remained agglutinated for a longer time than the sperm in sea water. Although the results showed wide variation, the contrast between the two sperm suspensions was quite striking. From the results, it seemed reasonable to conclude that seminal fluid had changed the sperm surface in such a way as to bring about a stronger reaction with agglutinin.

DISCUSSION

The seminal fluid factor and sperm motility

Gray (1928a) observed that the motility of the sperm of *Echinus miliaris* was in no way impaired when suspended in seminal fluid, and he stated conclusively that the seminal fluid possessed no chemical or physical properties inhibiting sperm motility. He prepared the seminal fluid, which he called "testicular plasma," by strong centrifugation of the "dry sperm," the same method employed in this investigation.

The experiments and observations of the present study confirm Gray. The earlier results of the work, given in a preliminary note (Hayashi, 1940), showed that the sperm of *Arbacia punctulata* were motile in seminal fluid, with an intensity of movement at least equal to that exhibited by sperm in sea water. Moreover, this motility persisted for a longer time in the former medium. That sperm are active in seminal fluid was confirmed by respiration studies (results to be given in a subsequent report), for it was found that the respiratory activity of sperm was maintained

at a higher level for a longer time in seminal fluid than in sea water. Therefore, it may be stated conclusively that sperm cells of *A. punctulata* and *E. miliaris* are fully active in seminal fluid.

The observations and conclusions of Southwick (1939a) were found to be in conflict with these results. This worker found that sperm of *Echinometra subangularis* were immobile when suspended in the seminal fluid of the same species. He concluded that there was present in the seminal fluid a substance which inhibited the activity of the sperm.

Hartman (1940) and Hartmann, Schartau, and Wallenfels (1940) confirmed Southwick on the presence of the inhibiting factor not only in the seminal fluid, but also in the sea water that had contained large numbers of spermatozoa. Their work, however, was done with the sperm of *Arbacia pustulosa*. In addition to confirming Southwick, Hartmann et al. stated that the function of the inhibiting factor was to neutralize echinochrome A, a sperm-activating substance from the egg.

For several reasons, the conclusions of these workers do not seem to be justified. In the first instance, Southwick's own observations reveal that freshly-exuded "dry sperm" possess an intense vibratory activity, an apparent contradiction to his own conclusion. This activity is lost after a few minutes. A number of investigators have published observations pertinent to these phenomena. Thus, Harvey (1930) showed that sperm of *Arbacia punctulata* in oxygen-free sea water were immobile; when oxygen was introduced the sperm regained their motility. Lillie (1913) demonstrated that sperm of Nereis and Arbacia lost their motility in the presence of carbon dioxide. Dungay (1913), using Nereis and Arbacia, Fuchs (1914) with *Ciona intestinalis,* and Cohn (1918) with *A. punctulata* proved that acid media had a deleterious effect on sperm. Finally, Carter (1931) working with *Echinus esculentus* and *Echinus miliaris,* and Taylor and O'Melveny (1941) with *Strongylocentrotus purpuratus* and *Lytechinus anamesus* obtained experimental proof that acid conditions lowered the respiratory activity of sperm.

In view of the results of these investigators, the brief activity of the sperm noted by Southwick seems to be attributable to the newly-made contact of the sperm with oxygen upon shedding. The subsequent inactivation of the sperm has its probable explanation in the acid conditions induced by the carbon dioxide production of the sperm.

Furthermore, the papers of Southwick and the Hartmann school yield no figures on the pH of the media used by these workers, nor do their texts give any evidence that this factor had been controlled. In addition, the conclusions of Hartmann et al. concerning the effect of echinochrome have not been confirmed by the experiments of Tyler (1939b) and Cornman (1940, 1941). The former worker found that neither echinochrome nor spinochrome would stimulate the respiration of sperm of *S. purpuratus*. The latter showed that crystalline echinochrome did not increase the motility of the sperm of *A. punctulata*. The paradoxical results of Tyler and Cornman as opposed to Hartmann et al. may be attributed to species difference. However, it is clearly possible that echinochrome does not activate sperm. The non-existence of a sperm-activating function by echinochrome seems to weaken the argument for the existence of a substance neutralizing that activating factor.

Because of these considerations, the concept of a sperm-inhibitor in the seminal fluid seems to be questionable. In the light of parallel experimental results as re-

gards sperm motility and respiratory activity (Hayashi, unpublished), it is concluded that there is no inhibitor of sperm motility in the seminal fluid of *A. punctulata*. This conclusion does not deny the inhibiting effects of hydrogen ions, the influence of which on the increase of the life-span of the sperm has been shown to be insignificant. To restate the conclusion: excluding the hydrogen ion factor, there is no inhibitor of sperm motility in the seminal fluid of *A. punctulata*.

The seminal fluid factor in its relation to the activating capacity of the sperm

Various experiments have proved that spermatozoa suspended in seminal fluid retain their capacity to activate eggs longer than sperm cells suspended in sea water (Tables II, III, IV). The factor in the seminal fluid responsible for the effect is not found in the perivisceral fluid, the factor is not the pH of the medium, and the factor is heat-sensitive (Table III). The seminal fluid factor is also non-dialyzable and precipitable with ammonium sulfate (Table IV). On the basis of these results, it may be tentatively stated that the seminal fluid factor is protein. However, the usual protein tests have not been made, so that this conclusion cannot be drawn with any finality, even though the conclusion is strongly supported by positive micro-Kjeldahl analyses indicating protein content in the order of 2.5 mg. protein per cc. of seminal fluid.

The seminal fluid factor, if protein, may act on the sperm cells in several ways. The factor may serve as a source of nutrient for the sperm, it may act on the sperm through the agency of the colloidal osmotic pressure which its presence establishes in the seminal fluid, or it may act through adsorption on the sperm surface. It is necessary to consider these possibilities carefully, if the mechanism of the action of the seminal fluid factor is to be clarified.

The possibility of the seminal fluid factor's acting as a nutrient will be taken up more fully in a later publication on the effect of the seminal fluid on the respiration of sperm. The statement can be made here that these studies indicate that the factor does not act as a nutrient for the sperm. Likewise, the probable protein nature of the factor argues against the idea of nutrition, for the large size of the molecule would prevent its absorption by the sperm. The fact that seminal fluid contains no reducing sugar is further support for the belief that the seminal fluid affords no nutritive elements for the sperm cells.

The question of the effect of colloidal osmotic pressure in prolonging the functional life of the sperm cell is unsettled. Although the further experimental results on the surface activity of seminal fluid substance validate the conclusions drawn, it is admitted that the effects of colloidal osmotic pressure on sperm longevity is still an open question.

The experimental results given in Tables V and VI constitute support for the idea of surface-action of the seminal fluid factor. The data show that both the sperm surface and the seminal fluid factor are surface-active on glass, and they indicate the possible identity of the sperm-surface-substance and the seminal fluid factor.

The foregoing considerations point strongly to the conclusions that the seminal fluid factor is protein and that it is present both in the seminal fluid and on the sperm surface. Since the seminal fluid factor enables the sperm to retain their fertilizing function, it seems logical to infer that the seminal fluid protein plays a part directly in the fertilization process. The data of Tables VIII and IX give support

to this idea. The experiment of Table VIII reveals the fact that, with the same amounts of sperm, a higher percentage of activation of eggs is achieved by sperm in seminal fluid than by sperm in sea water. Since the experiment was so arranged that the insemination tests were made immediately after the mixing of each solution, the possibility that a large number of sperm in the sea water were immobilized seems unlikely. The conclusion most compatible with the results is that the individual spermatozoön in seminal fluid possesses a greater fertilizing capacity than his fellow in sea water. The mere act of dilution in sea water, therefore, seems to have removed a large part of the activating substance from the surfaces of the sperm cells, reducing their individual activating power.

The idea of variation of the activating power of the individual sperm cell was first expressed by Glaser (1915). He diluted sperm serially in sea water, and found that several sperm cells were required to activate one egg cell, even though only one spermatozoön was required to bring about the biparental effect. Lillie (1915) found that when he used the same method of dilution as did Glaser, the fertilizing power of the suspension was far less than an equal concentration of sperm in a suspension diluted in one step. Although these two workers disagreed in their conclusions, their results point to the validity of Glaser's interpretation, which is confirmed in the present study.

Table IX shows the relative fertilizing powers of suspensions in sea water and seminal fluid after they had been aged for 10 hours. If the results are compared to those of Table VIII, it will be seen that the fertilizing capacity of the seminal fluid sperm suspension was not affected by the aging period but that the fertilizing capacity of the sea water suspension was markedly reduced. There are two possible explanations for the enhanced difference in the activating power of the two suspensions, both of which probably contribute to the effect. It is possible that in the 10-hour aging period, large numbers of the sperm cells in the sea water suspension were immobilized, so that they could not penetrate the jelly envelope surrounding each egg. Thus, the number of sperm cells making actual contact with the egg surface was reduced. The final result would be a decreased percentage of activation. The second possibility is the conclusion derived from the analysis of Table VIII, namely, that a substance functional in activation was removed from the sperm surface. During the 10-hour period, this removal presumably continued, so that the activating power of the individual sperm cell was further reduced. Therefore, even if all the spermatozoa remained motile and capable of making contact with the egg, more sperm cells per egg would be required for activation, and the end results would be a decreased percentage of activation. The experiment, therefore, tends to support the idea of an egg-activating substance on the sperm surface, and, also, shows the close relationship between the motile activity and the activating power of the sperm cell.

Many investigators have postulated the existence of a substance on the surface of the sperm and considered that it was protein in nature. Buller (1902), from observations of the sperm of various echinids, reported that the sperm surface was surface-active, not only on glass, but also on air bubbles. Lillie (1913) discovered that in the presence of egg secretions, the male germ cells of Arbacia and Nereis became agglutinated. He concluded that the surface of the sperm cell was "sticky." The marked similarity of the agglutination phenomenon to an immunological reac-

tion may be taken to be a strong indication for the protein nature of the responsible agent on the surface of the sperm cell.

More direct evidence came from the work of Popa (1927). Using histochemical technique, this worker concluded that the surfaces of Nereis and Arbacia sperm were covered with a layer of lipo-protein.

Mudd, Mudd, and Keltch (1929) investigated the surface charge of the sperm cells of various echinids. Using the cataphoresis chamber, they reported that the sperm surface was negatively charged. This negativity they found to be increased after agglutination with egg-water. They concluded that their method made possible the detection of substances on the sperm surface.

Henle (1938) and Henle, Henle, and Chambers (1938) found that heat-sensitive antigens existed on the surface of sperm heads. Their work was done with mammalian sperm. Tyler and O'Melveny (1941) obtained rabbit anti-serum by injection of whole sperm of *S. purpuratus* and *L. anamesus*. The anti-serum was found to agglutinate the sperm of these species. These immunological studies again pointed to the protein nature of the sperm-surface-substance.

The evidence cited is not, perhaps, a complete list. The investigations provide enough experimental data, however, to warrant the tentative conclusion that the sperm-surface-substance is protein in nature.

This sperm-surface-substance and the seminal fluid factor may possibly be identical (Tables V and VI). A strong indication of identity could be established if it were shown that the seminal fluid factor alone can activate eggs. Experiments have been started to investigate this possibility, but as yet no conclusive results have been obtained. Comparable work in this direction has not been done. The effect of protein extracts on egg surfaces was investigated by Favilli (1932) and by Hartmann, Schartau, and Wallenfels (1940), while Sampson (1926) reported the activation of eggs by dialysates and filtrates of sperm suspensions. Since her activating factor was dialyzable, and therefore non-protein, it cannot be compared to the seminal fluid factor. In addition, the remarks of Just (1922, 1928, 1929a and b) criticizing the auto-parthenogenesis of Glaser (1914) and Woodward (1921) are equally applicable to the work of Sampson.

Another possible method of establishing the identity of seminal fluid protein and the sperm-surface-substance is to obtain rabbit anti-serum by the injection of seminal fluid. If the anti-serum thus obtained had the power to agglutinate sperm, the results would constitute substantial evidence for the argument that seminal fluid protein and protein on the sperm surface were the same substance. The experiment, however, was not done.

The identity, therefore, is not established, although there is some evidence in this direction (Tables V and VI). Aside from this point, however, there are experimental results throwing light on the origin of and possible relation between the seminal fluid factor and the sperm surface substance. It would be interesting to know whether these substances are secreted by the sperm cells or not, and whether the sperm-surface substance establishes the seminal fluid substance by passing off into the seminal fluid, or whether the seminal fluid substance establishes the sperm-surface substance by adsorption on the sperm surface.

Numerous investigators have reported that sea-urchin sperm give off a substance into the surrounding sea water (Lillie, 1914 and 1915; Southwick, 1939; Hartmann,

1940; Hartmann, Schartau, and Wallenfels, 1940) and that this substance showed protein characteristics (Frank, 1939; Tyler and O'Melveny, 1941). All investigators agreed that the substance showed the properties of "antifertilizin" or the power to "bind" the agglutinin of egg-water so that the agglutinating effect on sperm was reduced.

The question here posed is: does this substance from the sperm cells have the properties of the seminal fluid substance? The point was tested by an experiment in which a sea water suspension of sperm was allowed to stand for several hours, the sperm cells removed by centrifugation, and a fresh sample of sperm suspended in the medium. The results were negative. This medium was not effective in prolonging the fertilizing capacity of sperm, and therefore did not have the properties of the seminal fluid substance.

There is the converse question: Does the seminal fluid substance have the antifertilizin property of the substance coming off the sperm cell? Again, the results were negative (Table X).

The substance coming off the sperm cell does not have the properties of the seminal fluid substance. The results of these experiments indicate, therefore, that a sperm substance does not establish the seminal fluid substance, so that the seminal fluid factor does not have its origin in the sperm cell. The same negative answer as to the origin of the sperm-surface substance cannot be given.

However, the fact that the substance coming off the sperm surface has different properties from the seminal fluid substance signifies nothing regarding the properties of the sperm substance while on the sperm surface. This substance on the surface of the sperm cell is surface-active on glass, as is the seminal fluid substance (Tables V and VI). The seminal fluid factor also enables the sperm cell to maintain its fertilizing capacity longer (Tables II, III, IV) and seems to enhance the fertilizing power of the individual spermatozoön. In addition, the seminal fluid factor affects the surface of the sperm so that the time of agglutination, or the reaction with agglutinin, is increased (Table XI).

These facts point to a tentative explanation of the relation between the seminal fluid factor and the sperm-surface substance. It is possible that a protein substance, originally present in the seminal fluid, is adsorbed on the surface of the sperm cell, thus influencing the fertilizing power of the sperm cell, as well as rendering the surface of the sperm cell surface-active. By this adsorption also, the sperm surface is rendered capable of greater reactivity with fertilizin. The subsequent loss of this substance from the sperm cell results in the loss of fertilizing power and the presence of antifertilizin in the sperm medium. The antifertilizin would be, according to this scheme, a substance changed in certain properties from the original seminal fluid substance.

Summary

1. A factor is present in the seminal fluid of *Arbacia punctulata* which prolongs the fertilizing capacity of the sperm cells of the same species.

2. The factor, which is not found in the coelomic (perivisceral) fluid, is heat-sensitive, precipitated by saturation with ammonium sulfate, non-dialyzable, and surface-active on glass. Since micro-Kjeldahl analysis of the seminal fluid gives positive results corresponding to 2.5 mg. protein per cc. of seminal fluid, it is tentatively suggested that the factor is protein.

3. Seminal fluid has a pH range of 7.6 to 7.9, its osmotic pressure is approximately 10 per cent lower than sea water, and its content of reducing sugar is negligible.

4. In equivalent concentration and immediately after suspension the fertilizing capacity of the individual spermatozoön is greater in seminal fluid than in sea water.

5. Seminal fluid does not contain antifertilizin since it does not neutralize the agglutinating action of egg-water; indeed, this action is intensified.

6. A tentative mechanism, based on the adsorption of a fertilizing substance and its removal from the surface of the sperm cell, is suggested to explain the experimental results. It is proposed that the seminal fluid factor is this fertilizing substance before adsorption and while on the surface of the sperm; it becomes changed upon removal from the sperm surface.

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HETEROCINETA PHORONOPSIDIS SP. NOV., A CILIATE FROM THE TENTACLES OF PHORONOPSIS VIRIDIS HILTON

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Introduction

The infestation of the tentacles of *Phoronopsis viridis* Hilton by a small ciliate of the family Ancistrocomidae Chatton and Lwoff [1] (order Holotricha, suborder Thigmotricha) was called to my attention by Professor Harold Kirby. A preliminary study of this ciliate, from slides prepared in his laboratory from material collected in Bodega Bay, California, in November, 1943, disclosed that on the basis of the organization of the ciliary system it appeared to be most closely related to species of the genus *Heterocineta* Mavrodiadi, ectoparasitic on fresh water mussels, prosobranchs, and pulmonates (Jarocki; 1934, 1935).

In June, 1945, I collected additional material of *Phoronopsis viridis* [2] in an intertidal mud flat in Tomales Bay. From observations of the living ciliates it was determined that this new species, which will be described herein as *Heterocineta phoronopsidis* sp. nov., differs fundamentally from other species of *Heterocineta* in having a groove-like depression originating on the left side of the body near the anterior end and extending posteriorly along the dorsal surface close to the left margin. I have studied a species of *Heterocineta* ectoparasitic on *Physa cooperi* Tryon from a locality near Mt. Eden, California, which agrees perfectly with the description of *Heterocineta janickii* given by Jarocki (1934). This ciliate, like *H. phoronopsidis,* has eight ciliary rows, but these are restricted to a more narrow area on the ventral surface. There is no dorso-lateral groove in *H. janickii.* In none of Jarocki's descriptions of ciliates of the genus *Heterocineta,* which apparently were based to a large extent upon living material, is there any mention of such a groove.

Technique

For observation of the living ciliates the tentacles of *Phoronopsis viridis* were detached from the rest of the body by means of forceps and comminuted in a drop of sea-water on a slide. Fixation of the organisms for permanent preparations was accomplished by preparing smears in this manner on coverglasses and dropping them onto the surface of the fixative in a Petri dish. For a study of the general

[1] Chatton and Lwoff (1939) proposed the family Ancistrocomidae to include those ciliates formerly assigned to the family Hypocomidae Bütschli which differed from the type genus of the latter (*Hypocoma* Gruber) in having the suctorial tentacle disposed terminally rather than subterminally and the ciliary rows arranged singly rather than in pairs.

[2] Professor W. A. Hilton of Pomona College has kindly identified the phoronid species from Tomales Bay as *Phoronopsis viridis* Hilton (1930). It should here be noted, however, that no systematic revision of the phoronids from the Pacific Coast has been given in the literature and it is not impossible that *P. viridis* will later be shown to be identical with one of the species described earlier.

morphology, staining with iron hematoxylin gave good results on material fixed in Schaudinn's fluid. Differentiation of the ciliary system by impregnation with activated protein silver (protargol) was successful following fixation in Hollande's cupric-picro-formol mixture and Schaudinn's fluid, but this method was no more satisfactory than staining with iron hematoxylin. The Feulgen nuclear reaction was used after fixation in Schaudinn's fluid and a saturated aqueous solution of mercuric chloride with 5 per cent of glacial acetic acid.

HETEROCINETA PHORONOPSIDIS sp. nov.

The body is elongated, asymmetrical, and flattened dorso-ventrally. Twenty living individuals taken at random ranged in length from 26 μ to 37 μ, in width from 11 μ to 16 μ, and in thickness from 6.5 μ to 11 μ, averaging about 29 μ by 14 μ by 8 μ. As seen in dorsal view (Fig. 1A) the left side of the ciliate is conspicuously rounded, while the right side is by comparison very little curved. The body is usually widest at a point a short distance behind the middle and is rounded posteriorly. The attenuated anterior end is deflected toward the left, truncate at the tip, and bent ventrally. The reduced ciliary system, to be described presently, is disposed in a shallow concavity occupying the anterior four-fifths of the ventral surface (Fig. 1B); the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex.

A contractile suctorial tentacle enables the ciliate to attach itself to epithelial cells of the tentacles of the host and to feed upon their contents. When fully extended the suctorial tentacle of *Heterocineta phoronopsidis* is about 4 μ in length and 1.5 μ in diameter; it is contracted as soon as the ciliate is dissociated from the host and is seldom preserved in an extended condition in fixed individuals except those which have been fixed in a position of attachment to the host.

The internal tubular canal (Fig. 1, c) continuous with the suctorial tentacle is about 1.5 μ in diameter in its anterior portion, which is directed dorsally, and becomes abruptly narrower in its posterior portion, which is directed ventrally and obliquely to the right. In some living specimens and in suitable preparations stained with iron hematoxylin the canal can be traced along the right side of the body to a point a short distance posterior to the macronucleus.

The cilia of *Heterocineta phoronopsidis* are about 5 μ in length and markedly thigmotactic. They are disposed in eight longitudinal rows limited to the shallow concavity on the ventral surface (Fig. 1C). All eight rows originate near the base of the suctorial tentacle. Each of the first five rows from the right margin is about three-fifths the length of the body. The fourth and fifth rows are as a rule practically straight, while the outer three are appreciably curved. The remaining three rows become progressively longer and inflexed in such a way that they end one behind the other near the mid-line. The eighth and longest row terminates at a point about four-fifths the distance from the anterior end of the body to the posterior end. The cilia of the anterior part of the thigmotactic system move rather actively, those of the posterior part sluggishly.

The shallow groove-like depression which distinguishes *Heterocineta phoronopsidis* from other species of *Heterocineta* has its inception on the left side of the body near the anterior end and is about four-fifths the length of the body (Fig. 1A, g). As it extends posteriorly it comes to lie on the dorsal surface along the left margin.

The groove is visible only in living individuals. There are no traces of ciliature at any point along its course. Staining with iron hematoxylin and impregnation with protein silver fail to bring out any basal granules in the region occupied by the groove.

The cytoplasm is colorless and contains a number of small refractile granules in addition to food inclusions. The refractile granules (Fig. 1A, cg) are apparently lipoid droplets, as they are dissolved out by toluol used for clearing following staining. At least one large food-vacuole and usually several smaller ones are present near the posterior end of the body (Fig. 1, fv). The contents of the food-vacuoles

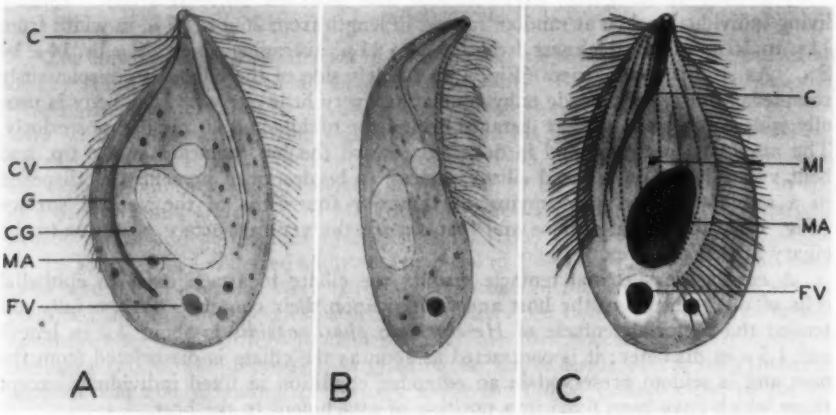

FIGURE 1. *Heterocineta phoronopsidis* sp. nov.

A. Dorsal aspect, from life; B. lateral aspect from right side, from life; C. ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida. × 1940.

c = internal tubular canal, cg = cytoplasmic granule, cv = contractile vacuole, fv = food vacuole, g = dorso-lateral groove, ma = macronucleus, mi = micronucleus.

are seen to consist mainly of ingested nuclei or fragments of nuclei from the epithelial cells of the tentacles of the host.

The contractile vacuole (Fig. 1, cv) lies near the middle of the body and opens to the exterior on the ciliated ventral surface. I have not distinguished a permanent opening in the pellicle.

The oval or rod-shaped macronucleus (Fig. 1, ma) is placed dorsally near the center of the body, its longitudinal axis lying obliquely to the longitudinal axis of the body. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction on the macronucleus ranged in length from 5.25 μ to 7.5 μ and in width from 3 μ to 4.5 μ.

The fusiform, rod-shaped, or crescentic micronucleus (Fig. 1C, mi) is situated anterior to the macronucleus. It is very difficult to distinguish in living specimens and is stained only weakly by iron hematoxylin and the Feulgen nuclear reaction. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction

the micronucleus ranged in length from 1.5 μ to 2.25 μ and in width from 0.75 μ to 1.2 μ.

When attached to the tentacles of the host *Heterocineta phoronopsidis* is almost immobile, exhibiting only a passive vibratory motion due to the energetic movement of the epithelial cilia. When dissociated from the host the ciliate swims slowly, usually rotating on its longitudinal axis and tracing wide arcs with its attenuated anterior end.

Heterocineta phoronopsidis sp. nov.

Diagnosis: Length 26 μ–37 μ, average about 29 μ; width 11 μ–16 μ, average about 14 μ; thickness 6.5 μ–11 μ, average about 8 μ. The anterior end is attenuated, bent ventrally, and provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary rows are eight in number and originate near the base of the suctorial tentacle. The first five rows from the right are about three-fifths the length of the body, while the remaining three rows become progressively longer and are inflexed in such a way that they end one behind the other near the mid-line. A groove-like depression, without any trace of ciliature, extends from the anterior end of the body posteriorly along the dorsal surface close to the left margin. Ectoparasitic on the tentacles of *Phoronopsis viridis* Hilton (Tomales Bay, California). Syntypes are in the collection of the author.

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ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1945

The role of bacteria in the excystment of the ciliate Didinium nasutum. C. D. Beers.

Resting cysts were obtained by the completion-culture method, viz., by preparing small cultures in spring water with paramecia as food. Most of the didinia in such cultures encysted upon exhaustion of the food supply. Such cysts never became active spontaneously, nor were they bacteria-free.

Distilled water, sugars, salts of plant acids, pH changes, and metabolites of Paramecium were ineffective in inducing excystment.

Timothy and lettuce infusions, and peptone and yeast-extract solutions induced 78–94 per cent excystment within 9–12 hours at 28° C. The tentative conclusion that these substances were effective excystment-inducing agents *per se* was soon negatived by the observation that at the time of excystment, bacteria (introduced with the cysts) were always flourishing in the media, which had been originally sterile. To test more adequately the effect of bacteria, these same four media were inoculated with wild bacteria from Paramecium cultures and incubated 18–24 hours. The bacterized media when tested on cysts induced 89–95 per cent excystment within 3–4 hours at 28° C. and thus produced a distinct acceleration effect.

The special effectiveness of bacterized peptone suggested an examination of the role of amino acids in excystment. Nine such acids were tested, singly and in mixtures, in buffered solution, but none yielded an accelerative effect. Only those acids (e.g., histidine, arginine, proline, methionine) and mixtures which supported bacterial growth induced excystment, and then only when bacteria were flourishing, i.e., after 9–12 hours. Acid mixtures previously inoculated with bacteria produced the usual accelerative effect. Hydrolyzed peptone behaved similarly.

Boiling the bacterized acid mixtures, or peptone solutions or hydrolyzates destroyed their effectiveness, which, however, could be restored by inoculation with bacteria.

The results indicate that excystment in Didinium is induced through the agency of bacterial action. Further studies are in progress to identify the effective bacteria, and to ascertain the chemical nature of the substances responsible for excystment.

Cytological studies in Culex. C. A. Berger and Sister Mary Grell.

Cells in the hind-gut of Culex ($2n = 6$) are diploid at the beginning of larval life and are highly polyploid at pupation. This polyploid condition arises by repeated chromosome reduplication within the resting nucleus. During metamorphosis these cells undergo mitotic division. The first division of a 16-ploid cell is described. Unique cytological features of this division are as follows. There are six groups of chromosomes each composed of eight sister chromosomes. Homologous groups are paired, relationally coiled and apparently have their spindle attachment regions fused. In early prophase the association of sister chromosomes is so close that the eight appear as one. As prophase contraction proceeds the eight sister chromonemata become evident and are seen to be relationally coiled in two's, in pairs of two's, etc. The spindle attachment region undergoes successive division in late prophase. At metaphase 48 chromosomes can be counted. Anaphase separation is regular and homologous or sister chromosomes pair as they move to the poles. This work can be interpreted as favoring the first part of Darlington's hypothesis, that chromosomes are attracted in pairs only, but gives no support to the second part of the hypothesis, that pairs of pairs repel.

Accelerating metamorphosis in the tunicate, Styela partita. Lloyd M. Bertholf.

C. Grave discovered that metamorphosis in tunicate larvae can be hastened by dozens of different substances, from complicated extracts of tunicate and vertebrate tissues down to simple salts of several heavy metals, added to sea-water. He concluded that such acceleration

is caused by a poisoning of the larval action-system, so that the adult action-system takes over sooner than normally, and that the chief agent in this poisoning is copper.

To ascertain how specific the need for copper is, an effort was made to hasten metamorphosis by various substances in which copper is absent. Isotonic solutions of NaCl alone or in combination with other salts and with lactose and sucrose were first used. All these solutions brought about metamorphosis much sooner than in the controls, provided the larvae were about 4 hours old or older; if younger, the animals usually died before metamorphosis or shortly afterward.

It is possible, however, that the salts used contained a threshold amount of copper and other heavy metals as impurities. Hence distilled water alone was next used. This killed the animals after a few minutes of continuous exposure, but if larvae of about 2 hours or older were immersed in distilled water for only ½ to 2 minutes and then transferred to normal sea-water, metamorphosis was much hastened, and no deleterious effects resulted.

It seems, then, that the effect of copper is not specific, but that similar effects can be produced by other means, including the physical shock of a large change in osmotic pressure.

Oxidation-reduction studies on Penicillium notatum and other organisms. Matilda Moldenhauer Brooks.

Redox potential and pH measurements by means of the Coleman electrometer were made of the media in which *Penicillium notatum* and several other organisms were grown. Daily readings were taken for a period of several weeks. *Penicillium* was grown in corn steep medium. *Aspergillus flavus, Mycoderma, Torula utilis* and *Sacchromyces cervesiae* were grown in modifications of Czapek-Doz media. Sterile conditions were maintained.

It was found that rH values ($= 2\ pH + E_h/.03$) for *Penicillium* were 8.4 to 8.7. For purified penicillin (100,000 Oxford units) the rH was 8.7. For other organisms it was either higher or lower. In the case of *Penicillium*, the E_h value became very negative (-0.25) and the pH alkaline (8.5). No other organism studied had these characteristics.

When flasks were tightly stoppered, the rH values were similar to those obtaining in cultures to which KCN had been added. Growth was hindered when aerobes were used and not affected in the case of facultative anaerobes.

It is suggested that the therapeutic action of penicillin and related organisms depends upon the balance between E_h and pH in the blood, which these organisms produce. This factor makes it incompatible for such organisms as *Staphylococcus aureus*, for example, to exist.

Organization of the giant nerve fiber system in Neanthes virens. Theodore H. Bullock.

The presence of giant nerve fibers in certain polychaete annelids has been known on the basis of anatomical studies, but their function and functional organization have not been investigated. The group is especially suitable for such studies since its members present a great diversity of neural development; giant fibers are present in varying pattern in many species, absent in others; the group is large, and favorable species for laboratory study are common. A survey of the functional anatomy of the giant system in representative forms has been undertaken to the end of adding perspective to our picture of the evolution of the nervous system and with the hope of finding material for special studies of nervous physiology. The electrical signs of nervous activity were used as a tool for revealing the functional anatomy.

The present report will be confined to *Neanthes virens* (*Nereis virens*). When the nerve cord is directly stimulated by single shocks there is recorded from the nerve cord or from the mid-ventral line of the intact animal, in any other part of the worm, a pattern of large spikes, several orders of magnitude higher in voltage than the action currents representing spontaneous activity of the small fibers of the nerve cord. These large spikes have the properties of single fiber action currents. The first is the largest, has the lowest threshold, fatigues the slowest, and arrives at a time representing a minimum conduction rate (assuming no delays) of 5 meters/sec. Unlike the others it is not all or none, but all or half or none; two independent units are present conducting at just the same rate but separable by threshold and fatigue. The second spike is intermediate in height, threshold, fatigue, and rate (4.5 m/s) between each half of the first spike and the later ones. A small third spike at 2.5 m/s may be alone or followed by another

like itself. This pattern is constant from specimen to specimen and may be regarded as characteristic of the species. One can expect anatomically at least four giant fibers or conducting units: a pair larger than the rest but identical in average diameter; a single unit, next in size; and one or two small but still "giant" units. This corresponds precisely with the known anatomy, there being a pair of large lateral fibers, a smaller median unpaired fiber, and a pair of still smaller medially placed fibers. The present technic can assure certain relations difficult to establish histologically. There is no anastomosis between the lateral fibers such as occurs in *Lumbricus;* the fibers are all independent conducting units, none being a necessary efferent or afferent connection of another; all the fibers are unpolarized, conducting equally well in both directions (although segmental macrosynapses like those in *Lumbricus* have been described). The sensory connections of each fiber can be inferred from responses to mechanical stimuli. The giants can each be fired through sense organs by local stimulation of the skin (a light tap or dropping water) within certain segmental levels; the head is not necessary. The median fiber (second spike with direct electrical stimulation) is fired by stimuli in the anterior quarter, approximately; the smallest, slowest giants by stimuli in the posterior three quarters and a region of overlap of a few segments occurs. The fast lateral giants can be fired from any level but require stronger stimuli (water dropping from a few cms. higher for example). The evidence suggests a function as mediators of startle responses to three classes of stimuli—weaker anterior, weaker posterior, and stronger at any level (differences in threshold in different levels exist for each fiber within this scheme). The two laterals usually fire together but in certain cases they can be separated.

The plan in general is very like that in *Lumbricus* although the two belong to different classes and many polychaetes with just as close a relation have no or very differently organized giant systems.

The displacement of terns by gulls at the Weepecket Islands. Sears Crowell.

The changes in population at the colony of Common and Roseate Terns at the Weepecket Islands are described for a period of twenty years. The colony attained, by 1931, a population of 3500 adult terns. During the past ten years this colony of terns has gradually been replaced by breeding Herring Gulls. The terns are probably incapable of successful reproductive activity if gulls are near, even though the latter do no direct injury to the terns.

The members of the Weepecket colony have been redistributed among other colonies of southern Massachusetts as shown by recoveries of banded birds.

Conditions favor a recolonization by the terns if the gulls are evicted or abandon the islands.

The influence of drugs on heat narcosis. A. Froehlich.

When the temperature of the surrounding water is slowly raised aquatic animals, such as crustaceae, fishes, tadpoles and frogs, show complete loss of voluntary and reflex muscular activity at a "critical point" of temperature which is characteristic for each species. This condition is reversible; transference into cool water causes the animals to recover promptly. "Heat narcosis" resembles narcosis brought about by drugs (alcohol, ether, etc.) in every way, except that where the former increases oxygen consumption, the drugs diminish oxygen consumption.

For reasons too numerous to mention here, I decided to investigate the influence of theophylline (as theophylline natrio-aceticum) on the "critical point" of heat narcosis. The experiments were performed on *Fundulus heteroclitus* at the M.B.L. in Woods Hole during the summer months of 1944 and 1945 and at the May Institute for Medical Research, Cincinnati, Ohio, on field frogs and tadpoles during the winter and spring of 1944–1945.

Theophylline given subcutaneously or intramuscularly in doses which had no visible effect on the behavior of the experimental animals produced a considerable lowering of the "critical point" in heat narcosis. The same effect was obtained if the animals were placed in a weak solution of theophylline.

Theophyllinized animals died much sooner than did controls if access to air was restricted. The water in which such animals died showed far greater acidity due to accumulation of CO_2.

Asphyxiation alone produced a lowering of the "critical point" similar to that obtained with theophylline.

Methylene blue (intramuscularly to Fundulus) produced effects on the "critical point," susceptibility to asphyxiation and acidity of the water which were similar to those obtained with theophylline.

In the experiments with theophylline as well as in those with methylene blue, previous conditioning in a 1 : 100,000 solution of quinine sulfate counteracted to a greater or a lesser degree the expected lowering of the "critical point."

It can be concluded that the action of theophylline and methylene blue on these experimental animals is, in part at least, to increase the demand for oxygen, and that quinine reverses this action by decreasing respiratory metabolism.

As I had previously found (with E. Zak) that an important part of the action of theophylline consists in increasing tissue permeability, I feel justified now in assuming that this phenomenon is caused by a condition of hypoxemia and acidosis (local asphyxia) in the tissues.

Reactions of oyster (Ostrea virginica) to free chlorine. Paul S. Galtsoff.

By measuring the rate of flow of water through the gills and by recording the shell movements it was possible to demonstrate that both the pumping mechanism of the oyster and its adductor muscle are very sensitive to free chlorine. In many oysters the first treatment with the concentrations as low as 0.01 or 0.02 p.p. million causes complete cessation of current and closure of shells, although there are specimens in which complete cessation of pumping and closing of shells takes place only in the concentrations approaching 0.5 p.p.m. Repeated treatments develop increased tolerance and pumping may be resumed at the concentrations much stronger than those which produced strong initial effect. Pumping, however, is not maintained at the concentrations of one p.p.m. or greater.

Variation in the sensitivity and development of tolerance are apparently associated with the secretion of mucus which provides protective coating for tentacles, mantle, and gills. Observations with a strobotac show that lateral cilia of the excised gill filaments continue to beat even at the concentration of 3 p.p.m. The cessation of pumping activity of an intact organism is due, therefore, not to the failure of the lateral cilia, but to the reaction of the regulatory mechanism of the pallium, which prevents the entrance of water to the gills, and to a certain extent to the disturbance of the rhythm of ciliary motion over the entire ciliated surface of the demibranches.

The presence of free Cl in water may materially impede the purification of oysters. It is therefore necessary that water, sterilized by chlorination and used in a process of purification, contains no residual Cl.

Development of granule-free fractions of Arbacia eggs. Ethel Browne Harvey.

A granule-free fraction of the *Arbacia punctulata* egg is obtained by breaking the egg with centrifugal force into two halves, and then breaking the lighter (white) half into two quarters, one of which contains all the remaining granules; and the other, the "clear quarter," is free of all granules visible in the living egg. This clear quarter contains the oil, nucleus, and most of the matrix or ground substance, but no mitochondria, yolk, or pigment. When fertilized, this clear quarter in many cases throws off a fertilization membrane, cleaves quite regularly, forms a perfect blastula and gastrula and pluteus. This pluteus may be quite normal with gut and skeleton, and later develops pigment spots, but is much clearer and less granular than that from the white half. It is approximately half the size of the pluteus from the white half and quarter the size of the pluteus from the whole egg. There is a considerable delay throughout development beginning with first cleavage, in spite of the fact that two nuclei (♂ and ♀) are present with a small amount of cytoplasm. The visible granules of the egg are therefore not necessary for development. The important substance in the cytoplasm is the ground substance or matrix, which is optically empty in the living state.

In vivo and in vitro glycogen utilization in the avian nematode Ascaridia galli. W. Malcolm Reid.

Glycogen constitutes one-third or more of the dry weight of many parasitic nematodes and flatworms. Extensive *in vitro* experiments upon glycogen utilization have been carried out by different investigators chiefly upon mammalian nematodes, cestodes, and trematodes. Von

Brand with *Ascaris lumbricoides* showed that 45 per cent of the glycogen reserve was utilized by females during 48 hours. Recent experiments upon fowl nematodes and cestodes have shown a much higher rate of glycogen utilization when the host had been starved for a short time. In a typical experiment with *Ascaridia galli*, 75 per cent of the glycogen reserve was utilized in 48 hours by female worms. With the fowl castode, *Raillietina cesticillus*, this reserve was depleted even more rapidly, 94 per cent of the glycogen being utilized in 24 hours.

Until a study using simultaneously *in vivo* and *in vitro* methods upon the same parasite has been completed, a comparison of the results of such experiments can have little meaning. Furthermore, such a study would serve as a check upon the earlier *in vitro* experiments which need re-examination now that improvements in technique have brought some of these results under question.

Glycogen determinations were made upon three groups of *A. galli*. Group I were controls and consisted of worms which were removed from the host after a normal feeding period. Group II worms were starved within the host for 48 hours before glycogen determinations were made. Group III consisted of parasites removed from the same hosts used for Group I, but these parasites were starved anaerobically for 48 hours at 41.5° ± 1° C. in one per cent saline using the same *in vitro* methods that were used on mammalian forms. Separate determinations were made on both males and females since sex differences in glycogen content were known to exist. The mean glycogen content for approximately ten samples for each group expressed in per cent of the wet weight of the worms is as follows: Group I females, 4.66; Group II females, 1.16; Group III females, 1.01; Group I males, 3.81; Group II males, 0.43; and Group III males, 0.26. The similarity in the rate of glycogen utilization with both males and females under the two conditions probably indicates that the *in vitro* methods used by early investigators reflect reliable information about normal glycogen metabolism within the host. Comparison between the glycogen utilization in the avian *A. galli* with the mammalian *A. lumbricoides* indicates that the much higher utilization rate in *A. galli* is real and not due to differences in technique.

Balanced centerwell solutions for manometric experimentation with cyanide. W. A. Robbie.

It has been demonstrated, both experimentally and theoretically, that the potassium cyanide-potassium hydroxide absorption solutions recommended by Krebs (1935, *Biochem. Journ.*, **29**: 1620) are not in hydrogen cyanide equilibrium with the experimental fluids for which they were designed. It is possible, however, to prepare, on the basis of experimental determinations, potassium cyanide-potassium hydroxide mixtures which will absorb carbon dioxide and maintain hydrogen cyanide equilibria with cyanide solutions of 0.011 M or less. The hydrogen cyanide tension of calcium cyanide solutions saturated with calcium hydroxide varies only with the concentration of the calcium cyanide and the temperature. This type of centerwell mixture will absorb carbon dioxide effectively and maintain equilibrium with hydrogen cyanide solutions up to 0.01 M.

*Studies of the muscle twitch recorded by electronic methods.** Alexander Sandow.

Piezoelectric, cathode-ray oscillographic methods have been devised for recording the various mechanical changes of the isometric twitch of skeletal muscles. To register the latency relaxation, LR (the minute precontractile elongation of a stimulated muscle during the latter half of the latent period), the apparatus is used, in effect, as an electronic lever which converts the LR into a 500,000 × magnified deflection on the cathode-ray screen. The piezoelectric pulse corresponding to the main contraction and relaxation periods is electronically differentiated and thus at each instant the cathode-ray deflection for this record is proportional to the rate of tension change in the course of the twitch.

These methods have been used to study the effect of maximal tetani of lengths from ⅕ to 10 sec. on the mechanical features of the twitch of the frog sartorius. The results prove that the separate processes that underlie the LR, the use of tension, and the post-contractile relaxation, are each uniquely affected by the tetanic activity. E.g., a 2 sec. tetanus causes a 10 per

* Supported in part by a grant from the Penrose Fund of the American Philosophical Society.

cent increase in the maximum rate of tension rise in a twitch, but a 40–60 per cent increase in the maximum rate of relaxation. The great lability of the relaxation process associated with the new chemical environment induced by the activity is specially significant in indicating that relaxation is not passive but is chemically driven.

The LR shows certain temporal features like those of Brown's alpha-process, thus indicating that it is an external mechanical sign of the alpha-process. Detailed analysis of the effect of activity and of pH on the LR, especially in reference to the duration of the latent period, suggests that the latent period is an interval during which myosin-ATPase is splitting ATP, and leads to the inference, now being subject to further test, that the LR corresponds to the formation of an enzyme-substrate complex between myosin and ATP which provides a mechanism for directly energizing and activating the myosin for contraction.

Experimentally induced tumors in an insect. Berta Scharrer.

In *Leucophaea maderae*, a large Orthopteran, the recurrent nerve was cut at various levels. This nerve, which belongs to the stomatogastric nervous system, innervates the anterior portion of the alimentary canal as well as the salivary glands and their reservoir. Within ten days to several months after the operations tumors developed in organs innervated by the recurrent nerve. Frequent sites of tumorous growth were the anterior portion of the mid-gut and the salivary reservoir. In the fore-gut and in the salivary glands well developed tumors were relatively rare. Several types of control operations, such as allatectomy and castration in which the recurrent nerve had remained intact, did not cause the development of tumors. Some of the tumors obtained after the cutting of the recurrent nerve attained considerable sizes. Histologically they consist of layers of cells which show various degrees of abnormality. In advanced stages part of the cells break down into a debris of brown color. About 300 specimens, nymphs as well as male and female adults, with experimental tumors, were studied.

The origin of neurosecretory granules from basophil constituents of the nerve cells in fishes. Ernest Scharrer.

Neurosecretory granules do not appear to be formed in association with the Golgi apparatus or the mitochondria, but with the basophil constituents of the secreting nerve cells. Three modes of origin of the granules have been observed. In the preoptic nucleus of most fishes the granules originate in association with the peripherally located Nissl bodies. The latter diminish to the extent to which the acidophil neurosecretory granules increase. In a second type found in the preoptic nucleus of Ameiurus, Noturus, Centropristes, and others the nuclei of the secreting nerve cells show invaginations. These are filled with basophil cytoplasm which may contain acidophil granules. In a third type which is characteristic of the nucleus lateralis tuberis of catfishes, the acidophil granules originate within the nuclei of the cells, apparently at the expense of the nuclear chromatin. All three types may occur in the preoptic nucleus of Centropristes.

*Evidence of a metabolic effect by potassium in lowering the injury potential of invertebrate nerve.** Abraham M. Shanes.

The action of potassium on the injury potential of spider and blue crab nerve has been studied over a concentration range of one to 530 mM. When the magnitude of these potentials is plotted against the logarithm of potassium concentration, the relative effectiveness of low potassium concentrations in lowering the potential is found to be ½ that of concentrations above 30 to 40 mM. The data may be replotted on a log-log graph on the assumption that potassium is inactivating an enzyme, the active form of which is proportional to the resting potential. Two straight lines intersecting at 40 mM fit the data very well, the slope at lower concentrations being about ½ and at higher concentrations about one. This graph is like one which has been obtained for the effect of urethane on oxygen consumption in yeast and Arbacia; in this case the inhibitor is believed to act on two processes. The same interpretation may be applied to potassium.

* Aided in part by a grant from the American Academy of Arts and Sciences.

The similarity of potassium to an actual inhibitor is even stronger if consideration is given to the effect on activity. Only at a concentration corresponding to almost complete cessation of the process affected at low concentration does activity appreciably and suddenly decrease. Thus, in crab nerve, conduction ceases between 37 and 42 mM. Potassium and excitability is unaffected up to 37 mM.

The effect of low potassium concentrations is definitely correlated with the simultaneous inhibition of an aerobic metabolic process which supports the injury potential. In concentrations of 10 to 30 mM potassium eliminates ⅔ of this process—values corresponding closely to those obtained previously in frog nerve.

*Physical-chemical studies on chromosomal nucleoproteins.** Kurt G. Stern.

The object of this research is to determine the size and shape of desoxyribosenucleoproteins, isolated from cell nuclei, with the aid of such quantitative methods as ultracentrifugation, diffusion, electrophoresis, viscosity, x-ray diffraction, and similar techniques. In this cooperative study, S. Davis, P. Macaluso, S. C. Shen, and I. Fankuchen are collaborating with the writer.

Thus far, the desoxyribosenucleoproteins from the nuclei of chicken red blood cells and from calf thymus gland have been studied. Measurements in the analytical ultracentrifuge, in the diffusion apparatus, and in Ostwald viscometers, performed on solutions of these purified nucleoproteins in one M. NaCl, indicate a molecular weight of the order of two to three million and axial ratios varying from 35 : 1 to 100 : 1. The discrepancy of the results obtained with independent techniques casts considerable doubt on the suitability of this solvent, proposed by Mirsky and Pollister, with regard to the native state of the nucleoproteins. It appears that these conjugated proteins are appreciably dissociated in M.NaCl-solution. According to preliminary experiments, one M.glycine appears to be a solvent better suited for physical-chemical studies on these macromolecules.

The theory that these desoxyribosenucleoproteins are capable of assuming a more or less *helical shape* in solution as a function of the nature and ionic strength of the solvent, is advanced as a working hypothesis. Thus it is assumed that these molecules reflect in their configuration, on a molecular scale, the coiling and uncoiling of the chromosomes of which they represent important constituents. Plastic models, constructed in accordance with this hypothesis, were demonstrated at the Seminar.

Action of quitenine on the livers of tautog and toadfish. Charles H. Taft.

When quinine is treated with potassium permanganate the vinyl group is oxidized to a carboxyl group yielding quitenine.

It has been shown (Dauber, M., 1920; *Zeit. für Expt. Path. u. Therapie*, 21 : 311) that quitenine had a damaging action on kidney tubules. Taft and Place (1944; *Texas Reports on Biol. and Med.*, 2 : 61) showed that quitenine was more injurious to the kidneys of a glomerular fish than to the kidneys of an aglomerular fish.

Quitenine dihydrochloride in a 0.25 molar solution was injected subcutaneously into the side of the fish. The doses used were 1, 2, and 4 mM/Kg. Fish were killed by a blow on the head after varying intervals of time. The livers were placed in Bouin solution. Sections were cut 6 μ thick.

On gross examination a few tautog livers were abnormally soft. Gall bladder was a greenish blue in all cases. In the toadfish the liver was soft in a few cases. Color of gall bladder ranged from white through pale pink, orange, yellow green to green. Variation in color is probably due to variation in amount of bile pigment production or to oxidation of bile pigment. The von Kupffer cells were undamaged as were pancreatic cells of hepatopancreas.

Microscopic examination of toadfish liver shows fatty metamorphosis and some parenchymatous degeneration. Microscopic examination of the tautog liver showed fatty metamorphosis, albuminous degeneration, hydropic degeneration, and parenchymatous degeneration.

Quitenine is more damaging to the liver of the tautog than to the liver of the toadfish. The damage to the livers is not as severe as it was in the kidneys.

* This work was made possible by a grant of The Carrie S. Scheuer Foundation of New York.

Differences in sensitivity, hatchability curves, and cytological effects between Habrobracon eggs x-rayed in first meiotic prophase and metaphase. Anna R. Whiting.

Unlaid Habrobracon eggs were x-rayed in first meiotic prophase (diplotene) and in late metaphase and allowed to develop parthenogenetically. Those treated in prophase have 50 per cent hatchability at about 12,000 r (lethal dose about 45,000 r); give an exponential hatchability curve which tends to become linear when dose is fractionated; may show, after treatment, fragments or bridges or both in division I, in division II or in both. Those treated in late metaphase have 50 per cent hatchability at about 400 r (lethal dose about 2,000 r); give a linear hatchability curve which does not change with fractionation of dose; may show fragments but no bridges in division I, either or both in division II. All eggs treated in either stage with lethal dose develop at least to first cleavage (20 per cent continue to blastoderm); show bridges and sometimes fragments in cleavage. A correlation of chromosome form, movement, and tension at time of treatment with sensitivity and cytological effects exists which suggests that x-ray injury is due to direct "hits" on chromosomes, and that sensitivity is associated with degree of tension to which chromosomes are exposed during irradiation; that nature of chromosome changes is due to their form and proximity during treatment. Lethal dose is not lethal to the treated cell (oocyte) but to its descendents (embryo) since chromosome fragmentation is not lethal, loss of fragments is.

The problem of reversal of male haploidy by selection. P. W. Whiting.

Except for the almost sterile, highly inviable diploid males of the wasp Habrobracon obtained in experimental cultures, diploid males are unknown in the Hymenoptera, as also in rotifers, thrips, mites except Mesostigmata, aleurodids and iceryine coccids, and in the beetle Micromalthus. It is probable that all normal males in these groups are haploid and that male haploidy has been attained in an evolutionary sense not more than six or seven known times. One of these attainments, taking place in an ancestral hymenopteron probably in the early Jurassic, has come to involve the entire order. Three conditions characterize male haploidy: (1) Production of males from reduced unfertilized eggs. (2) Reduction or omission of meiosis in spermatogenesis. (3) Complementary sex determination with heterozygous "double dominant" females. The problem of reversal of male haploidy is not to attempt to re-integrate any Jurassic protohymenopteran species, but rather to obtain by methods of genetics a strain of *Habrobracon juglandis* with normal biparentalism of males as well as of females. Inbreeding gives diploid males homozygous for sex. Selection has increased their viability from one to sixty per cent as compared with females. Cell size of diploid males is abnormally large, but is reduced somewhat in strains of high viability. Spermatogenesis of diploid males is of the haploid type, lacking chromosome synapsis and resulting in diploid sperm. If a strain with chromosome synapsis can be derived, it is considered that the problem can be solved, since sex determination should then shift from the complementary to the back-cross type with digametic females.

Endomitosis in plants. E. R. Witkus.

The process called endomitosis was discovered by Geitler in 1939 in insect material. During this process there is a chromosomal reduplication without a nuclear division, no spindle is present and there is no true anaphase movement of chromosomes. Throughout the whole process the nuclear membrane remains intact. Geitler divided the process into four stages, which he termed endoprophase, endometaphase, endoanaphase, and endotelophase. During endoprophase the chromosomes become shorter and thicker. The stage at which the chromosomes have reached their highest degree of contraction is called endometaphase. The nuclear membrane is intact and the chromosomes are not aligned on an equatorial plate. The SA-region of the chromosomes divides and the chromatids or now endoanaphase chromosomes slightly separate. After this separation the chromosomes undergo reversion to the resting stage. This reversion process occurs during endotelophase. The resulting cell then is tetraploid.

This process was also found to occur in the tapetal cells of *Spinacia oleracea* (Spinach) and apparently this is the first time that endomitosis, as defined by Geitler, has been reported for plant material.

The tapetal cells of *Spinacia* undergo two successive divisions during the early prophase stages of meiosis. The first division is an incomplete mitotic division resulting in binucleate

cells or in cells having dumb-bell shaped nuclei. The second of these divisions is in all cases endomitotic.

It becomes increasingly apparent that polyploidy brought about by a chromosomal reduplication without a nuclear division is of quite common occurrence in both plant and animal material. Endomitosis is only one of three known methods by which this can occur, although it has often been confused with all three in recent cytological literature. The first method is by a repeated reduplication in the resting nucleus as illustrated in the multiple complex cells of mosquito. The second is simply by a double reduplication in the resting nucleus as shown by certain cells in the root tips of polysomatic plants such as *Spinacia*. The third is by endomitosis.

It is interesting also to note that polyploidy arises by different methods in the root tip and tapetal cells of *Spinacia oleracea*.

A tetrahedral framework for native proteins? Dorothy Wrinch.

It was suggested in 1936 that a fabric or atomic lamina is an essential element in the structure of native proteins and the lactim cyclol fabric was formulated as a working hypothesis (*Nature*, **137**: 411). Today four different types of fabric—all necessarily polypeptide fabrics—are open for discussion; the lactim and enol cyclol fabrics, the hydrogen-bridged linear polypeptide fabrics, and the fabrics in which cyclized polypeptides are interlocked by hydrogen bridges (Jordan Lloyd and Wrinch, 1936; *Nature*, **138**: 758; Astbury and Wrinch, *ibid.*, **139**: 798; Wrinch, 1940; *Phil. Mag.*, **30**: 64; 1941, **31**: 177). This idea of an atomic lamina or fabric as characteristic of native proteins has been widely accepted and adopted. It has now been used to interpret the x-ray intensities in a study of horse methemoglobin in crystalline form.

We wish now to suggest that these (hol) intensities (Perutz, 1942; *Nature*, **150**: 324) suggest not only laminae parallel to the c-planes (Boyes-Watson and Perutz, 1943; *Nature*, **151**: 714) but also a second set of laminae at approximately the tetrahedral angle to the first. For this and a number of other reasons, the hypothesis is put forward that the native protein unit (of which there may be one, two, or more in the native protein particle) is built on a tetrahedral framework, the possibility that the enveloping truncated tetrahedron of the framework may be an octahedron not being excluded. In the case of horse methemoglobin, this suggestion implies trigonality of the individual frameworks about an axis approximately normal to the c-planes; thus it offers an interpretation of the fact that the lattice points and c-face centers in these planes form a triangular network which is very nearly equilateral. The hypothesis appears to bear very closely upon the fact that twins, trillings, and other compound crystals are extremely common in many different hemoglobins (Reichert and Brown, 1909; The Crystallography of the Hemoglobins, Washington, D. C.). This we propose for discussion the possibility that such situations as the apposition of tetrahedral frameworks by displacements plus rotations of (1) $\frac{1}{8}$, $\frac{3}{8}$ about the (111) axis; or (2) $\frac{1}{6}$, $\frac{1}{2}$, $\frac{5}{6}$ about the (111) axis; or (3) $\frac{1}{2}$ about the $(\bar{1}10)$ or $(11\bar{2})$ axes, or $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$ about the (100) axes, etc., are here realized. Attention is also directed to the obvious manner in which this postulate lends itself to the interpretation of the space groups and classes of crystal symmetries found in x-ray (Fankuchen, 1941; *Ann. N. Y. Acad. Sci.*, **41**: 157) or classical studies of the native proteins.